

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/228963137>

Methods in Malaria Research

Article · January 2008

CITATIONS

106

READS

4,158

5 authors, including:



[Hedvig Perlmann](#)

Stockholm University

122 PUBLICATIONS 5,286 CITATIONS

[SEE PROFILE](#)



[Mats Wahlgren](#)

Karolinska Institutet

428 PUBLICATIONS 10,559 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



Humoral and immunological memory among Ugandan subjects with uncomplicated malaria [View project](#)



Effects of sevuparin on rosette formation and cytoadherence of Plasmodium falciparum infected erythrocytes [View project](#)

METHODS IN MALARIA RESEARCH



Fourth Edition

edited by

Inger Ljungström, Hedvig Perlmann,
Martha Schlichtherle, Artur Scherf, Mats Wahlgren

MR4 / ATCC
Manassas, Virginia
2004

copyright © 2004
Inger Ljungström, Hedvig Perlmann, Martha Schlichtherle, Artur Scherf, and Mats
Wahlgren

copies may be obtained from:

Malaria Research and Reference Reagent Resource Center (MR4)
American Type Culture Collection
10801 University Boulevard, Manassas, VA 20110-2209

ISBN 0-930009-64-9

Cover created by Ewert Linder
Fourth edition, 2004

CONTENTS

Preface	ix
Contributors	xi
Abbreviations	xviii

PARASITES

I. Culturing of erythrocytic asexual stages of <i>Plasmodium falciparum</i> and <i>P. vivax</i>	
A. The candle-jar technique of Trager–Jensen	1
B. Establishment of long-term in vitro cultures of <i>Plasmodium falciparum</i> from patient blood	4
C. Short-term cultivation of <i>Plasmodium falciparum</i> isolates from patient blood.....	7
D. Growing <i>Plasmodium falciparum</i> cultures at high parasitemia	8
E. Arresting <i>Plasmodium falciparum</i> growth at the trophozoite stage with aphidicolin.....	9
F. Culturing erythrocytic stages of <i>Plasmodium vivax</i>	10
II. Freezing and thawing of asexual <i>Plasmodium</i> spp.	
A. Stockholm sorbitol method	12
B. Freezing of patient isolates and strains with glycerolyte	14
C. Thawing of glycerolyte-frozen parasites with NaCl	15
D. Thawing of cryopreserved <i>Plasmodium falciparum</i> using sorbitol	16
III. Staining of parasite culture or patient blood and estimation of parasitemia	
A. Acridine orange (AO) vital stain of cultures	17
B. Giemsa staining of thick or thin blood films	18
C. Estimation of the percentage of erythrocytes infected with <i>Plasmodium falciparum</i> in a thin blood film	20
D. Evaluation of parasitemia by lactate dehydrogenase assay	21
IV. Purification and synchronization of erythrocytic stages	
A. Enrichment of knob-infected erythrocytes using gelatine sedimentation	23
B. Sorbitol-synchronization of <i>Plasmodium falciparum</i> -infected erythrocytes	24
C. Enrichment of late-stage infected erythrocytes in 60% Percoll	25
D. Separation of <i>Plasmodium falciparum</i> mature stages in Percoll/sorbitol gradients	26
E. Obtaining free parasites	28
F. Obtaining semi-intact cells	29
G. Alanine synchronization of <i>Plasmodium falciparum</i> -infected erythrocytes	30
H. Selection of trophozoites by using magnetic cell sorting (MACS).....	31
I. Isolation of <i>Plasmodium falciparum</i> -infected erythrocytes from the placenta, see PARASITES section VII:A	33
V. Micromanipulation cloning of <i>Plasmodium falciparum</i>	
A. Micromanipulation cloning of parasites.....	34

B. Micromanipulation cloning of <i>Plasmodium falciparum</i> for single-cell RT-PCR ...	35
C. Expansion of <i>Plasmodium falciparum</i> clones	35
VI. Cytoadhesion and rosetting assays	
A. Basic cell media	37
B. Thawing melanoma and other cell lines	39
C. Freezing of cell lines	40
D. Cultivation of CHO, COS, HUVEC, melanoma, and L cells	41
E. Formaldehyde-fixation of melanoma cells	42
F. Binding assays to endothelial and melanoma cells	43
G. Selection of cytoadherent parasites by passage over C32 melanoma, CHO, HUVEC, or other endothelial cells	45
H. Binding of fluorescent receptors heparin, blood group A, and PECAM-1/CD31 to <i>Plasmodium falciparum</i> -infected erythrocytes	46
I. Adhesion of <i>Plasmodium falciparum</i> -infected erythrocytes to immobilized receptors	48
J. Adhesion of <i>Plasmodium falciparum</i> -infected erythrocytes to immobilized hyaluronic acid	51
K. Binding of <i>Plasmodium falciparum</i> infected erythrocytes to placental tissue sections, see PARASITES, section VII:B.....	52
L. Enrichment of rosetting parasites using Ficoll–Isopaque (Pharmacia)	53
M. Reversion of rosettes	54
VII. Placental malaria	
A. Isolation of <i>Plasmodium falciparum</i> -infected erythrocytes from the placenta	56
B. Binding of <i>Plasmodium falciparum</i> -infected erythrocytes to placental tissue sections.....	58
VIII. Detection of antibodies to the infected erythrocyte surface	
A. Reversion of rosettes, see PARASITES, section VI:M	61
B. Agglutination assay using purified trophozoite-infected erythrocytes. Measurement of antibodies to <i>Plasmodium falciparum</i> variant antigens on the surface of infected erythrocytes	61
C. Serum micro-agglutination of infected erythrocytes.....	63
D. Flow cytometry detection of surface antigens on fresh, unfixed red blood cells infected with <i>Plasmodium falciparum</i>	65
E. Analysis of plasma antibodies to variant surface antigens by flow cytometry.....	67
IX. Immunoglobulin- or serum protein-binding to infected erythrocytes	
A. Stripping erythrocytes of bound serum proteins and reformation of rosettes	69
B. Detection of serum proteins on the surface of <i>Plasmodium falciparum</i> -infected erythrocytes	71
C. Binding and eluting antibodies from the <i>Plasmodium</i> -infected RBC surface.....	73
D. Enrichment of immunoglobulin binding parasites using Dynabeads	75
X. Fixation protocols for transmission electron microscopy	
A. Fixation of tissues samples.....	77
B. Embedding samples for electron microscopy	79
C. Fixation of erythrocytes for immuno-electron microscopy	81

XI. In vitro reinvasion and growth inhibition assays	
A. In vitro reinvasion and growth inhibition assay by microscopy of erythrocyte monolayers	83
B. In vitro reinvasion and growth inhibition assay by flow cytometric measurement of parasitemia using propidium iodide (PI) staining	85
C. ³ H-hypoxanthine incorporation assay for the study of growth inhibition by drugs	87
D. <i>Plasmodium falciparum</i> hemozoin formation assay.....	90

MOSQUITOES and PARASITES

I. Rearing of <i>Anopheles stephensi</i> mosquitoes, see MOSQUITOES and PARASITES section II:C	93
II. Culturing of sexual, oocyst, and sporozoite stages	
A. <i>Plasmodium falciparum</i> gametocyte culture, purification and gametogenesis	93
B. Cultivation of <i>Plasmodium falciparum</i> gametocytes for mosquito infectivity studies	96
C. Production of <i>Plasmodium falciparum</i> oocysts and sporozoites.....	100
III. Transmission blocking and sporozoite invasion assay	
A. Transmission blocking assay (TBA) – membrane feeding.....	108
B. Sporozoite invasion assay	112

ANIMAL MODELS

I. Infection of monkeys with <i>Plasmodium</i> spp.	115
II. Infection of mosquitoes with <i>Plasmodium</i> spp. in monkeys.....	118
III. Culturing erythrocytic stages of <i>Plasmodium chabaudi</i>: a mouse malaria model	121

IMMUNOCHEMISTRY

I. Studies of the <i>Plasmodium falciparum</i>-infected erythrocyte surface	
A. Surface iodination of PRBC (Lactoperoxidase method)	123
B. Solubilization/extraction of surface-radiolabelled proteins	124
C. Alternative solubilization protocol.....	125
D. Immunoprecipitation of ¹²⁵ I-labelled PfEMP1	126
II. Metabolic labelling of <i>Plasmodium falciparum</i>	
A. Metabolic labelling of <i>Plasmodium falciparum</i> -infected erythrocytes.....	128
B. Immunoprecipitation using metabolically labelled parasite extracts	129
III. Resolution of giant proteins (200 kDa to 1 MDa) of <i>Plasmodium falciparum</i> on polyacrylamide–agarose composite gels	131

IV. Recovery of <i>Plasmodium falciparum</i> native proteins and active enzymes	
A. Solubilization in the presence of non-detergent sulphobetaines, mild solubilization agents for protein purification	132
B. Isoelectric focusing of proteins in a Rotofor cell: a first step in protein purification	133
SEROLOGY	
I. Antibody staining of <i>Plasmodium falciparum</i>-infected erythrocytes	
A. Erythrocyte membrane immunofluorescence (EMIF)	135
B. Erythrocyte membrane staining by enzyme-linked antibodies (EMEAS).....	137
C. Parasite immunofluorescence (PARIF)	138
II. Antibody selection on immobilized antigen or peptide	139
III. ELISA	
A. Equipment, materials, and reagents	140
B. Antigens for coating	142
C. ELISA procedure	143
CELLULAR IMMUNOLOGY	
I. Preparation of human peripheral blood mononuclear cells (PBMC)	145
II. Antigen preparations and peptides for cellular work	
A. Crude parasite antigen (Percoll-band).....	147
B. Desalting of synthetic peptides	148
III. T-cell proliferation	149
IV. Interleukin ELISPOT	150
V. Reverse transcription PCR for detection of cytokines.....	152
VI. Detection of cytokine-gene polymorphism	
A. Extraction of human DNA from peripheral blood or buffy coat.....	153
B. PCR for amplification of nucleotide repeat (VNTR) polymorphisms of cytokine genes.....	155
C. PCR for amplification of single nucleotide polymorphisms or RFLP of cytokine genes.....	156
D. Amplification Refractory Mutation System (ARMS) PCR for detection of previously characterised mutations in cytokine genes.....	157
VII. Haptoglobin phenotyping using PAGE.....	159
MOLECULAR BIOLOGY	
I. Small-scale genomic DNA isolation from <i>Plasmodium falciparum</i>	161

II. RNA extraction from <i>Plasmodium falciparum</i>	
A. RNA extraction from <i>Plasmodium falciparum</i> using guanidine thiocyanate and acidic phenol.....	163
B. Measurement of RNA yield and purity, and visualization on agarose–formaldehyde gel	167
C. Reliable RNA preparation from <i>Plasmodium falciparum</i>	169
D. Simple Northern blots	173
III. <i>Plasmodium falciparum</i> cDNA library construction	
A. Cell preparation at desired cell stage(s).....	176
B. RNA preparation (total RNA)	176
C. mRNA selection of pooled total RNA	176
D. cDNA synthesis	178
E. cDNA insertion, propagation, and amplification in phage using Pharmacia's λ ExCell Not I/EcoR I/CIP kit	179
IV. PCR of AT-rich genomes/regions.....	182
V. Single cell RT-PCR of <i>var</i>(DBL-1) mRNA from <i>Plasmodium falciparum</i>	183
VI. Fluorescent <i>in situ</i> hybridization (FISH)^{DNA-DNA} for <i>Plasmodium falciparum</i>.....	185
VII. Separation and mapping of chromosomes of malaria parasites using pulsed field gel electrophoresis (PFGE)	
A. PFGE Protocol 1	188
B. PFGE Protocol 2.....	193
VIII. Recombinant expression of <i>Plasmodium falciparum var</i>-gene domains..	196
IX. Transfection of <i>Plasmodium falciparum</i> within human red blood cells	
A. Materials	198
B. Transfection	199
C. Transfectant harvest and expression assays	200
X. Alternative protocol using DNA-loaded red blood cells for transfection ...	203
XI. Selectable episomal transfection of asexual stage <i>Plasmodium falciparum</i> parasites	205
A. Preparation of parasite cultures and DNA constructs	206
B. Electroporation of circular plasmid DNA into parasites	207
C. Maintenance of transfected parasite lines	208
XII. Telomere repeat amplification protocol in <i>Plasmodium falciparum</i> (PfTRAP)	212
XIII. Genotyping of <i>Plasmodium falciparum</i> parasites by PCR: <i>msp1</i>, <i>msp2</i>, and <i>glurp</i>	215

FIELD BLOOD SAMPLING

I. Finger-prick blood sampling	
A. Blood collection on filter paper.....	221
B. Blood collection in EDTA tubes	222
II. Serum extraction from filter-paper samples	223
III. DNA extraction	
A. DNA extraction from filter-paper samples	224
B. DNA extraction from filter-paper discs previously used for serum extraction	226
C. DNA extraction from EDTA-tube samples	227
D. Fast methanol-based DNA extraction from blood spots in filter paper.....	228
IV. Postdose drug level monitoring	230
Suppliers	231
Index	234

Preface

This is the fourth edition of *Methods in Malaria Research*. The first two were our “in-house” collections of protocols that we have found very useful in the lab. The fourth edition is a revised and expanded version of the third, which was produced with the help of the MR4 of ATCC, who also distributed the book online globally (www.malaria.atcc.org/publication.html). We hope you find our collection of malaria protocols useful.

You will find some innovations in this edition such as new chapters (animal models, mosquitoes), ten new sections, including more than 30 new protocols. New headings are found in each protocol such as Equipment and Materials and reagents. A list of contributors and the names and addresses of the authors of the various protocols will be of help if you have questions or comments about the methods.

In assembling this book, we have tried to maintain a broad angle on what protocols to include that are relevant to malaria research, be it culturing of parasites, immunological assays, or molecular biological techniques. One criterion we have set is that the methods be well-tried by those sending us protocols. We have intended to give enough detail in the methodology that someone who is not an expert in the field of malaria will still be able to make use of these protocols. Naturally we hope those of you who are expert malariologists will also find this book beneficial and interesting.

We acknowledge the work of Jane Edwards at ATCC for copyediting the text, writing the index, and compiling the lists of abbreviations and suppliers.

We consider this book a “working document” of malaria protocols that, with the help of our readers/users, will continuously grow and evolve as new and improved methods are communicated to us. If you find mistakes or have a method you think is suitable for the next edition, please write to:

Inger Ljungström, Swedish Institute for Infectious Disease Control,
SE-171 82 Solna, Sweden
e-mail: inger.ljungstrom@smi.ki.se

If you have comments or questions regarding the protocols please contact the authors directly.

Inger Ljungström, Hedvig Perlmann,
Marta Schlichtherle, Mats Wahlgren
Stockholm, Sweden
January 2004

Artur Scherf
Paris, France
January 2004

Contributors

Aydin Schmidt, Berit

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: berit.schmidt@smi.ki.se

Bakhsis, Nassera

Institut Pasteur, Unité BIHP, rue du Dr. Roux, 75015 Paris, France

Barragan, Antonio

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: antonio.barragan@smi.ki.se

Beeson, James G.

Department of Medicine, University of Melbourne, Post Office Royal Melbourne Hospital, Parkville VIC 3050, Australia

e-mail: beeson@unimelb.edu.au

Bereczky, Sandor

Malaria Research Laboratory, Department of Medicine, Karolinska Hospital, SE-171 76 Stockholm, Sweden

e-mail: sandor.bereczky@ks.se

Bhattacharyya, Mrinal Kanti

Kumar Lab, Johns Hopkins Malaria Research Institute, Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, USA

e-mail: mbhattac@jhsph.edu

Bolad, Ahmed

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: ahmed@imun.su.se

Bottius, Emmanuel

Institut Pasteur, Unité BIHP, rue du Dr. Roux, 75015 Paris, France

Braun-Breton, Catherine

Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur, 75724 Paris, France

e-mail: cbb@pasteur.fr

Chen, Qijun

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: chen.qijun@smi.ki.se

Collins, William E.

Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 4770 Buford Highway, Chamblee, GA 30341, USA
e-mail: wec1@cdc.gov

Deitsch, Kirk W.

Microbiology and Immunology, Joan and Sanford I. Weill Graduate School of Medical Sciences of Cornell University, New York, NY 10021, USA
e-mail: kwd2001@med.cornell.edu

El Ghazali, Gehad

Department of Clinical Immunology, Uppsala University Hospital, SE-751 85 Uppsala, Sweden
e-mail: gehad.elghazali@klinimm.uu.se

Färnert, Anna

Division of Infectious Diseases, Department of Medicine, Karolinska Institutet, Karolinska Hospital, SE-171 76 Stockholm, Sweden
e-mail: anna.farnert@medks.ki.se

Faye, Ingrid

Department of Genetics, Stockholm University, SE-106 91 Stockholm, Sweden
e-mail: ingrid.faye@genetics.su.se

Fernandez, Victor

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden
e-mail: victor.fernandez@smi.ki.se

Fidock, David A.

Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, The Bronx, New York, NY 10461, USA
e-mail: dfidock@aecom.yu.edu

Figueiredo, Luísa M.

Laboratory of Molecular Parasitology, Rockefeller University, 1230 York Avenue, New York, NY 10021, USA
e-mail: Luisa.Figueiredo@mail.rockefeller.edu

Flick, Kirsten

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: kirsten.flick@mtc.ki.se

Freitas-Junior, Lucio

Institut Pasteur, Unité BIHP, 25 rue du Dr. Roux, 75015 Paris-France
e-mail: junior@pasteur.fr

Gill, Pedro

Malaria Research Laboratory, Department of Medicine, Karolinska Hospital,
SE-171 76 Stockholm, Sweden
e-mail: pedro.gill@ks.se

Gyan, Ben

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden, and
Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana
e-mail: bgyan@noguchi.mimcom.net

Haeggström, Malin

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish
Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: malin.haeggstrom@mtc.ki.se

Hayano, Masashi

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden, and
Department of Tropical Medicine, Jikei University, Tokyo, Japan
e-mail: mhayano@imun.su.se

Heddini, Andreas

Multilateral Initiative on Malaria, Wenner-Gren Institute, Stockholm University,
SE-106 91 Stockholm, Sweden
e-mail: andreas.heddini@mim.su.se

Helmby, Helena

Immunology Unit, Department of Infectious and Tropical Diseases, The London
School of Hygiene and Tropical Medicine, London WC1E 7HT, UK
e-mail: helena.helmby@lshtm.ac.uk

Hernandez-Rivas, Rosaura

Department of Molecular Biomedicine, Centro de Investigación y de Estudios
Avanzados del I.P.N., México D. F., México
e-mail: rosaura36@hotmail.com

Hirai, Makoto

Department of Genetics, Stockholm University, Svante Arrhenius väg 16E, Room
E545, SE-106 91 Stockholm, Sweden, and
Department of Medical Zoology, Jichi Medical School, 3311-1, Minamikawachi,
Tochigi, 329-0498, Japan
e-mail: makoto.hirai@genetics.su.se

Hollingdale, Michael R.

The London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK
e-mail: michael.hollingdale@lstmh.ac.uk

Jouin, Helene

Unité d'Immunologie moléculaire des Parasites, CNRS URA 1960, Institut Pasteur,
75724 Paris, France
e-mail: hajouin@pasteur.fr

Kaneko, Akira

Tokyo Women's Medical University, Department of Tropical Medicine, Tokyo 162-
8666, Japan, and
Malaria Research Laboratory, Department of Medicine, Karolinska Hospital, SE-171
76 Stockholm, Sweden
e-mail: akirak@research.twmu.ac.jp

Kyes, Sue

Molecular Parasitology Group, Weatherall Institute of Molecular Medicine,
Headington, Oxford OX3 9DS, UK
e-mail: skyes@molbiol.ox.ac.uk

Kumar, Nirbhay

Kumar Lab, Johns Hopkins Malaria Research Institute, Department of Molecular
Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins
University, Baltimore, MD 21205, USA
e-mail: nkumar@jhsph.edu

Lebbad, Marianne

Swedish Institute for Infectious Disease Control and Microbiology and Tumor Biology
Center (MTC), Karolinska Institutet, SE-171 82 Solna, Sweden
e-mail: marianne.lebbad@smi.ki.se

Ljungström, Inger

Swedish Institute for Infectious Disease Control and Microbiology and Tumor Biology
Center (MTC), Karolinska Institutet, SE-171 82 Solna, Sweden
e-mail: inger.ljungstrom@smi.ki.se

Locker, Martin

School of Biology, University of Leeds, Leeds LS2 9JT, UK
e-mail: m.locker@leeds.ac.uk

Mann, Emily J.

Department of Medicine, University of Melbourne, Royal Melbourne Hospital,
Parkville VIC 3050, Australia
e-mail: e-mann@pgrad.unimelb.edu.au

Mattei, Denise

Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur,
75724 Paris, France
e-mail: dmm@pasteur.fr

McCormick, Christopher J.

School of Biology, University of Leeds, Leeds LS2 9JT, UK

e-mail: c.j.mccormick@leeds.ac.uk

Minang, Jacob

Faculty of Science, University of Buea, Cameroon

e-mail: jtminang@hotmail.com

Mok, Bobo

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish

Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: bobo.mok@mtc.ki.se

Nielsen, Morten A.

Centre for Medical Parasitology, Department of Infectious Diseases M7641,

Rigshospitalet, Copenhagen, Denmark

e-mail: mncmp@rh.dk

Nyakeriga, Alice

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden, and

Moi University FHS, Dept of Immunology, Eldoret, Kenya

e-mail: anyakeriga@hotmail.com

Perlmann, Hedvig

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: hedvig.perlmann@imun.su.se

Pettersson, Fredrik

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish

Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: fredrik.pettersson@mtc.ki.se

Rogerson, Stephen J.

Department of Medicine, University of Melbourne, Post Office Royal Melbourne

Hospital, Parkville VIC 3050, Australia

Scherf, Artur

Institut Pasteur, Unité BIHP, rue du Dr. Roux, 75015 Paris, France

e-mail: ascherf@pasteur.fr

Schlichtherle, Martha

PE Biosystems, SE-112 92 Stockholm, Sweden

e-mail: martha_schlichtherle@eur.appliedbiosystems.com

Scholander, Carin

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish

Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

Sidhu, Amar Bir Singh

Department of Microbiology and Immunology, Albert Einstein College of Medicine,
Forchheimer 403, 1300 Morris Park Avenue, The Bronx, New York, NY 10461, USA
e-mail: asidhu@aecom.yu.edu

Snounou, Georges

Unité de Parasitologie Biomédicale et CNRS URA 1960, Institut Pasteur, 25 Rue du
Dr. Roux, 75724 Paris Cedex 15, France
e-mail: snounou@pasteur.fr

Söderlund, Ankie

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden
e-mail: ankieso@hotmail.com

Staalsoe, Trine

Centre for Medical Parasitology, Department of Infectious Diseases M7641,
Rigshospitalet, Copenhagen, Denmark
e-mail: tscmp@rh.dk

Taylor-Robinson, Andrew W.

School of Biology, University of Leeds, Leeds LS2 9JT, UK
e-mail: a.w.taylor-robinson@leeds.ac.uk

Treutiger, Carl Johan

Center for Infectious Medicine (CIM), Karolinska Institutet, Huddinge Hospital,
SE-141 86 Stockholm, Sweden
e-mail: carl.johan.Treutiger@medhs.ki.se

Troye-Blomberg, Marita

Department Immunology, The Wenner-Gren Institute, Stockholm University,
SE-106 91 Stockholm, Sweden
e-mail: marita@imun.su.se

Udomsangpetch, Rachanee

Department of Pathobiology, Faculty of Science, Mahidol University, Thailand
e-mail: scrud@mahidol.ac.th

Vafa, Manijeh

Immunology Department, The Wenner-Gren Institute, Stockholm University,
SE-106 91 Stockholm, Sweden
e-mail: manijeh@imun.su.se

Vogt, Anna

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish
Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: anna.vogt@mtc.ki.se

von Euler, Anne

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish
Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: anne.von.euler@mtc.ki.se

Wahlgren, Mats

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish
Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: mats.wahlgren@mtc.ki.se

Wahlin-Flyg, Birgitta

Octapharma AB, SE-112 75 Stockholm, Sweden
e-mail: birgitta.wahlin-flyg@octapharma.se

Wu, Yimin

Malaria Vaccine Development Unit, NIAID, National Institutes of Health,
5640 Fishers Lane, Rockville, MD 20852, USA
e-mail: yiwu@niaid.nih.gov

Abbreviations

2-ME	2-mercaptoethanol (= β -mercaptoethanol)	NHS	normal human serum
AO	acridine orange	NPP	nitrophenyl phosphate
ATCC	American Type Culture Collection, Manassas, VA, USA	OD ₂₆₀	optical density at 260 nm
BrB	breaking buffer	PAGE	polyacrylamide gel electrophoresis
BSA	bovine serum albumin	PARIF	parasite immunofluorescence
CAT	chloramphenicol acetyl-transferase	PBMC	peripheral blood mononuclear cells
CGMRC	Centre for Geographic Medicine Research Coast	PBS	phosphate-buffered saline
CHEF	clamped homogeneous electric field	PCR	polymerase chain reaction
CLB	cell lysis buffer	PfEMP1	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
CPD	citrate phosphate dextrose	PFGE	pulsed-field gel electrophoresis
cpm	count per minute	PHA	phytohaemagglutinin
DEPC	diethyl pyrocarbonate	PMSF	phenylmethylsulfonyl fluoride
DMSO	dimethyl sulfoxide	PRBC	parasitized red blood cell(s)
DTT	dithiothreitol	RBC	red blood cell(s)
EDTA	editic acid; ethylenediamine-tetraacetic acid	RESA	ring-stage erythrocyte surface antigen
EGTA	ethylene glycol-bis(β -aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid	RT-PCR	reverse transcription polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay	SDS	sodium dodecyl sulfate
EM	electron microscopy	SSC	sodium chloride/sodium citrate (buffer)
EMEAS	erythrocyte membrane staining by enzyme linked antibodies	TAE	Tris/acetate (buffer)
EMIF	erythrocyte membrane immunofluorescence	TBA	transmission blocking assay
FBS	fetal bovine serum	TBE	Tris/borate (buffer)
FCS	fetal calf serum	TCM	tissue culture medium
FITC	fluorescein isothiocyanate	TE	Tris/EDTA (buffer)
GDA	glutaraldehyde	TEMED	<i>N,N,N',N'</i> -tetramethyl-ethylene-diamine
gDNA	genomic DNA	TEN	NaCl in TE buffer
HEPES	4-(2-hydroxyethyl)-piperazine-ethanesulfonic acid	TH	Tris-buffered Hanks' solution
HGG	human gamma globulin	TO	thiazole orange
HS	human serum	Tris	tris(hydroxymethyl)amino-methane
IPTG	isopropyl-1-thio- β -D-galactopyranoside	TSE	Tris/sodium chloride/EDTA solution
KEMRI	Kenya Medical Research Institute	USP	United States Pharmacopeia
LB	Luria broth	WB	washing buffer
MAP	multiple antigen peptide	WBC	white blood cell(s)
MCM	malaria culture medium	X-gal	5-bromo-4-chloro-3-indolyl- β -galactoside
MOPS	3-(<i>N</i> -morpholino)propane sulfonic acid		
MR4	Malaria Research & Reference Reagent Resources Center		
NDSB	nondetergent sulfobetaine		

PARASITES

I. Culturing of erythrocytic asexual stages of *Plasmodium falciparum* and *P. vivax*

I:A. The candle-jar technique of Trager–Jensen

by **Malin Haeggström¹**, **Martha Schlichtherle¹**, and **Ahmed Bolad²**

¹Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

²Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: malin.haeggstrom@mtc.ki.se, ahmed@imun.su.se

Equipment

incubator (37 °C)
glass desiccator (e.g., candle jar)
cell culture flask
candles
centrifuge
sterile pipettes
sterile tubes
glass slides and coverslips
microscope, fluorescence or light

Materials and reagents

purified erythrocytes (or human blood type O⁺ in CPD-adenine (Terumo) or S.A.G.M. ("Sagman" solution or EDTA)
MCM (see below)
Tris (Sigma)
Albumax II (Gibco)
RPMI 1640 (Gibco)
gentamicin
1 M HEPES (Gibco)
Hanks' balanced salt solution (Gibco)
acridine orange (10 µg/mL) or Giemsa 5%

optional:
human serum
glucose
hypoxanthine (Sigma)
Tris-buffered Hanks' (TH)

Preparations

Prepare malaria culture medium (MCM) and Tris-buffered Hanks' (TH) for washing cells.

Albumax complete medium

10.43 g RPMI 1640 powder (Gibco)
25 mL 1 M HEPES solution or 6 g HEPES (Gibco)
2 g NaHCO₃
0.5 mL gentamicin (from 50 mg/mL stock)

5 g Albumax II

Add distilled water to 1 liter. Filter-sterilize.

Use within 10 days, store at -20°C .

Comment: For growing parasites from patient blood, use 10 g of Albumax for 1 liter of complete MCM. The vast majority of cultures will survive at least 2 weeks. It is also important to avoid serum in the culture for preparation of crude parasite antigen (see SEROLOGY, section III:B). Not all strains can be adopted to Albumax II medium.

Alternative MCM

10.43 g RPMI 1640 powder (Gibco)

25 mL 1M HEPES or 6 g HEPES

2 g NaHCO_3

0.5 mL gentamicin (from 50 mg/mL stock)

Add distilled water to 1 liter. Filter-sterilize and store at -20°C in 45-mL aliquots.

For complete MCM (cMCM), add 5 mL of human blood type AB⁺ serum (inactivated at 56°C for 60 min; then stored at -20°C) to 45 mL of medium. Complete MCM can be used for up to one week if stored at 4°C .

MCM can also be made from commercial liquid RPMI with sodium bicarbonate and HEPES buffer (Gibco). Just add 5 mL of $100\times$ L-glutamine (Gibco) and 0.25 mL gentamicin (Gibco) to a 500 mL bottle of the RPMI.

TH (0.15 M Tris-buffered Hanks') (pH 7.2)

2.11 g Tris-HCl

0.2 g Tris-base

7.88 g NaCl

Dissolve in distilled water and bring volume to 1 liter.

Mix 1 volume of Tris buffer with 1 volume of Hanks'.

In vitro cultures in tissue-culture flasks

- Wash the erythrocytes 3 times in TH or RPMI 1640 to remove CPD, serum, and leukocytes if present. Dilute to 5% hematocrit with cMCM in small flasks of 25 cm^2 (0.2 mL of packed cells to 4 mL of cMCM) or in 75-cm^2 flasks (1.0 mL to 20 mL).
- Add parasites to an appropriate parasitemia (see below).
- Put the flask in a candle jar and loosen the screw cap. Produce low oxygen by burnt out candle and place the jar at 37°C .
- Replace the MCM every day (not necessary the day after subcultivation).
- Subculture the cultures 2 times/week.

Subcultivation

- Stain a drop of the culture with acridine orange ($10\text{ }\mu\text{g/mL}$) on a glass slide and put on a coverslip or by Giemsa staining of a thin smear (see PARASITES, section III:A or B).

- Count the parasitemia (i.e., the percentage of infected cells, see PARASITES, section III:C).
- Prepare freshly washed O⁺ blood in cMCM (5% hematocrit) and add it to the culture to obtain a parasitemia of not more than 1%, preferably 0.1 to 0.5% if two cycles until next subculturing, 0.5 to 1% if one cycle. Parasitemia should never exceed 15%.

References

Cranmer SL, Magowan C, Liang J, Coppel RL, Cooke BM. 1997. An alternative to serum for cultivation of *Plasmodium falciparum* in vitro. Trans R Trop Med Hyg 91(3):363-365.

Trager W, Jensen JB. 1976. Human malaria parasites in continuous culture. Science 193(4254):673-675.

I:B. Establishment of long-term in vitro cultures of *Plasmodium falciparum* from patient blood

by **Morten A. Nielsen** and **Trine Staalsoe**

Centre for Medical Parasitology, Department of Infectious Diseases M7641, Rigshospitalet, Copenhagen, Denmark

e-mail: mncmp@rh.dk

Careful adherence to the procedures described below should allow a very high success rate with immediate culture (provided parasites are not already damaged by antimalarial drugs), and even when using cryopreserved parasite stabilates, a success rate of >70% should be within reach.

Equipment

centrifuge

incubator

Materials and reagents

culture medium:

500 mL RPMI 1640 (Gibco)

25 mg gentamycin (Gibco)

91.6 mg L-glutamine (Sigma)

2.5 g Albumax II (Invitrogen) (See comments below.)

10 mL normal human serum

10 mg hypoxanthine (Sigma) (See comments below.)

washing medium:

500 mL RPMI 1640

25 mg gentamycin (Gibco)

freezing solution:

sterile, distilled water

3.0% sorbitol (Sigma)

28.0% glycerol (Sigma)

0.65% NaCl

thawing solution:

sterile, distilled water

3.5% NaCl

Giemsa stain:

10% Giemsa in phosphate buffer

gas mixture:

2% O₂, 5.5% CO₂, 92.5% N₂ (Special gas mixtures such as this one can be bought from suppliers of compressed gases.)

red blood cells (RBC) for subcultivation:

Blood type O Rh⁻ or O Rh⁺ RBC in CPD buffer, washed 3 times in washing medium to remove the Buffy coat (See comments below.)

Step-by-step manipulations

- Collect heparin or CPD anticoagulated venous blood from patient. Do not use EDTA blood, which does not support parasite survival. The collected blood can be used for cultivation immediately ("straight out of the arm") or after cryopreservation.

Preparation for immediate cultivation (“straight out of the arm”) (optional)

- Wash the anticoagulated blood sample 3 times in prewarmed (37 °C) washing medium (centrifuged for 8 min at 600 × g). Remove buffy coat after each centrifugation.
- Continue as described under *Cultivation of fresh patient isolates* below.

Cryopreservation (optional—Do only if immediate culture is not possible or desirable.)

- Spin the anticoagulated blood sample (centrifuged for 8 min at 600 × g) and remove plasma.
- Mix equal amounts of ring-stage parasitized packed RBC and freezing solution (see comments below).
- Distribute the mixture immediately into 1.8-mL screw-cap tubes and snap-freeze in liquid nitrogen.
- Store the tubes in liquid nitrogen until use.
- On day of use, thaw the RBC suspension in a 37 °C-water bath.
- Spin the tube (8 min at 600 × g) immediately after thawing (see comments below).
- Discard the supernatant, add an equal amount of thawing solution, mix well, and incubate the cells for 1 min (see comments below).
- Fill the tube with prewarmed (37 °C) washing medium and repeat centrifugation as above.
- Wash the RBC 2 times in prewarmed (37 °C) washing medium (centrifuge for 8 min at 600 × g).
- Continue as described under *Cultivation of fresh patient isolates* below.

Cultivation of fresh patient isolates

- Suspend RBC at ~4% hematocrit in prewarmed (37 °C) culture medium.
- Add 5 mL of the RBC suspension to a 50-mL cell culture flask (T flask) or 25 mL to a 250-mL cell culture flask.
- Flush 50-mL flasks for ~30 s with gas mixture (250-mL flasks for ~90 s) at 1.5- to 2-bar pressure (see figure and comments below).
- Incubate the flasks at 37 °C for 24 h.
- Remove the culture flask gently from the incubator and remove spent supernatant.
- Remove a tiny amount of RBC with a sterile Pasteur pipette and use these RBC to make a Giemsa-stained thin smear.
- Add new (prewarmed) culture medium to the flask, gas it as above, and return it to incubation at 37 °C.
- Subcultivate the parasites when necessary (see comments below).

Comments

- The Albumax II and hypoxanthine we use cannot be considered sterile. We thus make a stock solution of 100 g of Albumax II and 400 mg of hypoxanthine in 2 liters of RPMI 1640 and filter the solution through 0.8-μm and 0.2-μm filters. We then use 50 mL of this stock solution per 500-mL bottle of culture medium.
- It is important to keep all media preheated to 37 °C and to minimize handling time outside the 37 °C incubator. (No coffee breaks while handling cultures!)
- When gassing the culture flasks, fit the gas hose with a 0.2-μm filter unit, and use a sterile needle (preferably blunt to avoid accidents) (see the figure).

- If initial patient parasitemia is high ($>0.4\%$), change the medium the next day, otherwise leave it for 48 to 72 h before the first change of culture medium.
- Subcultivate patient isolates to keep parasitemia below 1 to 2% by adding fresh uninfected RBC. Although many laboratory-adapted parasite lines tolerate 5% parasitemia or more, this is not the case for most patient isolates.
- Do not leave parasites in freezing or thawing solution longer than absolutely necessary, as these solutions are harmful to the parasites.
- Washed uninfected RBC for subculture should be kept in the refrigerator for 24 h before first usage (to discourage any remaining leukocytes), and can be kept in the refrigerator for up to 14 days.

References

Cranmer SL, Magowan C, Liang J, Coppel RL, Cooke BM. 1997. An alternative to serum for cultivation of *Plasmodium falciparum* in vitro. *Trans R Soc Trop Med Hyg* 91(3):363-365.

Jensen JB, Trager W. 1977. *Plasmodium falciparum* in culture: use of outdated erythrocytes and description of the candle jar method. *J Parasitol* 63(5):883-886.

Nielsen MA, Staalsoe T, Kurtzhals JA, Goka BQ, Dodoo D, Alifrangis M, Theander TG, Akanmori BD, Hviid L. 2002. *Plasmodium falciparum* variant surface antigen expression varies between isolates causing severe and non-severe malaria and is modified by acquired immunity. *J Immunol* 168(7):3444-3450.

Trager W, Jensen JB. 1976. Human malaria parasites in continuous culture. *Science* 193(4254):673-675.

Figure



I:C. Short-term cultivation of *Plasmodium falciparum* isolates from patient blood
by **Malin Haeggström** and **Martha Schlichtherle**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: malin.haeggstrom@smi.ki.se

We give here a protocol for growing *P. falciparum* from patient blood. The vast majority of cultures will survive only the first cycle (i.e., from the ring stage to trophozoites/schizonts) and will not be able to reinvade new RBCs. In our hands, about 90% of patient isolates grew for at least one cycle. For establishment of long-term cultivation, see PARASITES section I:B.

Equipment

37 °C incubator
centrifuge for 500-3000 × *g*
candle-jar or gas (92% N₂, 5% CO₂, 3% O₂)

Materials and reagents

venous blood from malaria patient
heparinized or EDTA tubes
Polymorphprep (Axis-Shield)
RPMI 1640 (optimal HEPES-buffered)
O⁺ RBCs (or autologous blood group)
MCM with AB⁺ serum
culture flasks or plates

Procedure

- Collect 2 to 5 mL of venous blood into heparinized/EDTA tubes and keep at 4 °C. Process the sample within 1 hour.
- Carefully layer the blood over 5 mL of Polymorphprep and centrifuge at 500 × *g* for 15 min.
- Collect the cell layer of interest. Erythrocytes are in the bottom of the tube.
- Add 10 mL of sterile RPMI 1640 (37 °C) to the cells, resuspend, and centrifuge at 500 × *g* for 5 min.
- Aspirate the supernatant and repeat the wash twice.
- Estimate the pellet volume and mix with equal amount RPMI 1640.

If you wish to keep the monocytes, transfer the cell layer containing monocytes into a sterile 15-mL tube (see the manufacturer's protocol for Polymorphprep). Wash once with RPMI 1640 by centrifugation at 3,000 to 5,000 × *g* for 2 min. Remove the supernatant and add 1 mL of 10% DMSO in fetal calf serum. Freeze the cells immediately at −70 °C and then transfer to liquid nitrogen.

Reference:

Haeggström M, Kironde F, Berzins K, Chen Q, Wahlgren M, Fernandez V. 2004. Common trafficking pathway for variant antigens destined for the surface of the *Plasmodium falciparum*-infected erythrocyte. Mol Biochem Parasitol 133(1):1-14.

I:D. Growing *Plasmodium falciparum* cultures at high parasitemia**by Malin Haeggström and Martha Schlichtherle***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institutet for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: malin.haeggstrom@mtc.ki.se*

By using increasing volumes of culture medium, one can grow *P. falciparum* to higher parasitemia (above 10%). This protocol may be especially suited for maintaining high parasitemias of wild isolates, allowing maturation of a maximum number of ring stage parasites to trophozoites.

Procedure

- Set up cultures as described in PARASITES, section I:A, but use the table below as a guide for medium volumes.
- Increase the parasitemia gradually using the indicated volumes until a desired parasitemia is reached, then maintain the culture in the appropriate volume. For example, for a 200- μ L packed cell culture, use 4 times the volume of medium in the table for any given parasitemia.

% Parasitemia	Volume of medium (mL)/50 μ L of packed culture
10	2.5
12	3
14	3.5
16	4
18	4.5
20	5
24	6
28	7
32	8
36	9
40	10
44	11
48	12
52	13
56	14
60	15
64	16
68	17
72	18
76	19
80	20
84	21
88	22
92	23
96	24
100	25

Reference

KEMRI, CGMRC / Wellcome Trust Research Unit, Kilifi, Kenya

I:E. Arresting *Plasmodium falciparum* growth at the trophozoite stage with aphidicolin
by **Malin Haeggström**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: malin.haeggstrom@mtc.ki.se

This is a method to get fresh/frozen isolates or in vitro cultures to arrest in late trophozoite stages by inhibiting DNA synthesis.

Materials and reagents

malaria culture (only young stages! see PARASITES, section I:A) or a vial of frozen malaria parasites
aphidicolin (Sigma-Aldrich)
DMSO

Preparations

- Prepare a 1.5 mg/mL stock-solution of aphidicolin in DMSO.
- Dilute stock-solution 1:1000 into your malaria culture (4 µL in a 4-mL flask).
- Let the culture continue to grow until the parasites reach trophozoite stage (around 24 h after adding the aphidicolin). See PARASITES, section I:A.

Analyse or use your culture within 12 h after trophozoite stages are reached. Even though the parasites may look fine for some more hours, it is NOT recommended to use them after a longer time in aphidicolin.

Note: If you add the aphidicolin to a culture of later stages, the cells will not arrest.

References

Bull P, KEMRI, CGMRC, Kilifi, Kenya (personal communication)

Inselburg J, Banyal HS. 1984. *Plasmodium falciparum*: synchronization of asexual development with aphidicolin, a DNA synthesis inhibitor. Exp Parasitol 57(1):48-54.

I:F.Culturing erythrocytic stages of *Plasmodium vivax*by **Rachanee Udomsangpetch**

Department of Pathobiology, Faculty of Science, Mahidol University, Thailand

e-mail: scrud@mahidol.ac.th

Equipment

CF11-column (Cellulose powder CF11, Whatman)
syringe
centrifuge
orbital shaker (Leigh Labs)
fluorescence or light microscope

Materials and reagents

PBS
McCoy's 5A medium (Sigma–Aldrich)
reticulocyte-enriched red blood cells (may be obtained from cord blood or from hematochromatosis patients)
human AB serum
ethidium bromide (Sigma)
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
brilliant cresyl blue (ALD, Sigma–Aldrich)
slides and coverslips

Procedure

- Collect 5 mL of heparinised blood from *P. vivax*-infected patients. Prepare thin and thick blood films for examination of parasite stages and parasitemia.
- Dilute the blood with 5 mL of PBS.
- Pack a sterile CF11-column having the same volume as the diluted blood (10 mL) in a syringe and equilibrate it with PBS before use. Add the diluted blood to the column and elute the red blood cells (RBC, reticulocyte enriched) with PBS. The leukocytes will stick to the CF11 cellulose.
- Collect the RBC passing through the column (reticulocyte enriched), centrifuge at 2,000 rpm, and wash once in McCoy's 5A medium at room temperature.
- Resuspend the RBC to 5% hematocrit in McCoy's 5A medium containing 25% human AB serum and maintain the culture in a candle jar or use a gas mixture containing 5% CO₂, 5% O₂, 90% N₂.
- In order to enhance growth of parasites, shake the culture gently and continuously at 75 rpm in the orbital shaker.
- Check the blood smears daily to confirm maturation of the parasite and add reticulocyte-enriched RBC when required.
- Enumerate the infected RBC using one of the following techniques:
 - Mix 1 drop of ethidium bromide (10 µg/mL) with one drop of the culture, mount the mixture on a glass slide with a coverslip, examine it under a UV-light microscope, and count the number of the parasitised RBC from 10,000 RBC.
 - Mix 1 volume of 1% brilliant cresyl blue in 5 volumes of the culture. Mount and examine as described above.

References

Chotivanich K, Silamut K, Udomsangpetch R, Stepniewska KA, Pukrittayakamee S, Looareesuwan S, White NJ. 2001. Ex-vivo short-term culture and developmental assessment of *Plasmodium vivax*. Trans R Soc Trop Med Hyg 95(6):677-680.

Udomsangpetch R, Socci R, Williams J, Sattabongkot J. Modified techniques to establish a continuous culture of *Plasmodium vivax* [abstract]. 2002. In: American Society of Tropical Medicine and Hygiene 51st Annual Meeting program; 2002 Nov 10-14; Denver, CO.

II. Freezing and thawing of asexual *Plasmodium* spp.

We give several methods below for cryopreserving and thawing *Plasmodium* strains and wild isolates. In general, parasites frozen according to one method can be thawed using another method's thawing protocol. For convenience, we generally freeze parasite strains using the Stockholm Sorbitol Method, while thawing using the simpler NaCl Method or the 5% & 27% Sorbitol Method. It appears that the lengthier thawing protocol of the Stockholm Sorbitol Method gives lower initial parasitemia (killing of the later stages) and a more synchronous culture during the first two cycles.

When parasites are cultured in Albumax II MCM to avoid adhering IgG, no addition of serum is required.

II:A. Stockholm Sorbitol Method

by **Malin Haeggström** and **Martha Schlichtherle**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: malin.haeggstrom@mtc.ki.se

Equipment

tabletop centrifuge (Beckman)
liquid nitrogen tank
immufuge

Materials and reagents

fetal bovine serum (Gibco)
freezing medium:
28% glycerol
3% sorbitol
0.65% NaCl
distilled water
To make 250 mL: Mix 180 mL of 4.2% sorbitol in 0.9% NaCl with 70 mL of glycerol. Filter-sterilize; store frozen.

Freezing

- Transfer a 4-mL culture into a test tube.
- Pellet erythrocytes by centrifugation (immufuge on low for 1 min) and discard the supernatant; the pellet is approximately 0.2 mL.
- Add 0.3 mL of serum (of complementary blood group) (40% hematocrit).
For Albumax II cultures (see PARASITES, section I:A), add 0.3 mL of fetal bovine serum (40% hematocrit).
- Add 0.5 mL of the freezing medium, drop-by-drop, while shaking the vial gently; the addition should take approximately 1 min.
- Transfer the medium into a sterile cryovial.
- Drop the vial gently into the liquid nitrogen tank.

Thawing

- Prepare the sorbitol solutions listed below, have MCM and heat-inactivated serum ready (see PARASITES, section I:A). For Albumax II MCM, see also the comment above.

- Take a malaria culture vial from the liquid nitrogen tank.
- Thaw it quickly, approximately 1 min in a 37 °C-water bath.
- Put the vial on ice and transfer the contents into chilled 50-mL centrifuge tubes and centrifuge gently to pellet the cells ($250 \times g$ for 5 min at 4 °C in a Beckman tabletop centrifuge).
- Remove the supernatant and add the following, drop by drop, while shaking the vial gently:
 - 1 mL 17.5% sorbitol in ice-cold PBS, then
 - 2 mL 10% sorbitol, and finally
 - 2 mL 7.5% sorbitol
- Centrifuge as before, remove the supernatant, then add:
 - 1 mL 10% sorbitol, then
 - 2 mL 7.5% sorbitol, and finally
 - 2 mL 5% sorbitol
- Centrifuge, then add:
 - 1 mL 7.5% sorbitol, then
 - 2 mL 5% sorbitol, and finally
 - 2 mL 2.5% sorbitol
- Centrifuge, then add:
 - 1 mL 5% sorbitol, then
 - 2 mL 2.5% sorbitol, then
 - 2 mL MCM with 15% serum (at room temperature)
- Centrifuge, then add:
 - 1 mL 2.5% sorbitol, then
 - 2 mL MCM with 15% serumTransfer to a 5-mL tube.
- Centrifuge in the immufuge.
 - Add 4 mL of MCM containing 15% serum (see comment for Albumax II MCM above) and packed erythrocytes of a complimentary blood group (see also PARASITES, section I:A).

II:B. Freezing of patient isolates and strains with glycerolyte

by **Malin Haeggström** and **Martha Schlichtherle**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: malin.haeggstrom@mtc.ki.se

This protocol is useful for all primate malaria parasites tested (*Plasmodium falciparum*, *P. vivax*, *P. knowlesi*, *P. coatneyi*, *P. fragile*, and *P. cynomolgi*).

Cryoprotective solution

glycerolyte:

57% glycerol USP

16 g/liter of sodium lactate

300 mg/L of KCl USP

25 mM sodium phosphate (pH 6.8)

(This solution may be purchased from Baxter/Fenwal.)

Procedure

All steps are at room temperature unless otherwise stated.

- Take heparinized blood containing very young ring stages of malaria and centrifuge it for 10 min at $700 \times g$ (approximately 3,000 rpm).
- Remove the supernatant and estimate volume of packed cells, V.
- SLOWLY add $0.33 \times V$ of glycerolyte through gentle mixing. Let the tube stand for 5 min.
- Add dropwise $1.33 \times V$ of glycerolyte, mixing gently.
- Label cryotubes with the parasite's code/name and date.
- Distribute 1 mL of the preparation per cryovial.
- Freeze at -70°C for at least 18 h.
- Transfer vials to liquid nitrogen for long-term storage.

II.C. Thawing of glycerolyte-frozen parasites with NaCl

by **Malin Haeggström** and **Martha Schlichtherle**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: malin.haeggstrom@mtc.ki.se

Materials and reagents

sterile 1.6% NaCl (Baxter/Fenwal, 4B7870)

sterile 12% NaCl (Baxter/Fenwal, 4B7874)

malaria culture medium (MCM, see PARASITES, section I:A)

15% AB⁺ serum

Procedure

- Remove a vial from cold storage and thaw it at 37 °C for 1 to 2 min.
- Transfer blood to 50-mL centrifuge tubes with a sterile pipette. Measure blood volume, V.
- Add $0.1 \times V$ of 12% NaCl slowly, dropwise, while shaking the tube gently.
- Let the tube stand for 5 min.
- Add $10 \times V$ of 1.6% NaCl slowly, dropwise, swirling the tube.
- Centrifuge the tube at $500 \times g$ at 20 °C for 5 min.
- Aspirate the supernatant and add $10 \times V$ of MCM slowly, dropwise, while shaking the tube.
- Centrifuge the tube at 1,500 rpm at 20 °C for 5 min and aspirate the supernatant.
- Resuspend pelleted blood cells in MCM with 15% AB⁺ serum and transfer to a culture flask and desiccator. For continued culturing, see PARASITES, section I.

II:D. Thawing of cryopreserved *Plasmodium falciparum* using sorbitol**by Malin Haeggström and Martha Schlichtherle***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: malin.haeggstrom@mtc.ki.se***Equipment**

water bath (37 °C)
table-top centrifuge (Beckman)

Materials and reagents

27% sorbitol in PBS, filter-sterilized, stored at 4 °C
5% sorbitol in PBS, filter-sterilized, stored at 4 °C
15% AB⁺ serum

Procedure

- Remove a vial from liquid nitrogen and thaw it quickly in a 37 °C water bath; shake it lightly.
- Transfer thawed parasites into a round-bottomed 13-mL tube (max. 1 mL/vial).
- Slowly add 2 volumes of 27% sorbitol, the first volume over 8 min, the second over 5 min. Slowly stir the tube constantly on a vortex.
- Let it stand for 5 min.
- Add 2 volumes of 5% sorbitol over 10 min.
- Let it stand for 5 min.
- Centrifuge the tube at $400 \times g$ (1,250 rpm on a table-top Beckman centrifuge) for 3 min; discard the supernatant.
- Add 2 volumes of 5% sorbitol over 8 min.
- Let it stand for 5 min.
- Centrifuge the tube at $400 \times g$ for 3 min; discard the supernatant.
- Add 1 to 2 mL of MCM over 1 min. Centrifuge the tube at $400 \times g$ and discard the supernatant. The wash may be repeated once.
- Resuspend the pellet in 15% AB⁺ serum in MCM. Add washed, packed RBC's to 5% hematocrit.
- Change the medium the next day. Continue culturing as usual (see PARASITES, section I).

Reference

Procedures received from M. Hommel, modified from:

Diggs C, Joseph K, Flemmings B, Snodgrass R, Hines F. 1975. Protein synthesis in vitro by cryopreserved *Plasmodium falciparum*. Am J Trop Med Hyg 24(5):760-763.

III. Staining of parasite culture or patient blood and estimation of parasitemia

III:A. Acridine orange (AO) vital stain of cultures

by **Malin Haeggström**¹, **Martha Schlichtherle**¹, and **Hedvig Perlmann**²

¹Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: malin.haeggstrom@mtc.ki.se

²Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: hedvig.perlmann@imun.su.se

Equipment

UV-equipped microscope

Materials and reagents

Acridine orange (AO) (Fluka)

Stock solution:

Dissolve 10 mg of AO in 10 mL of distilled water. Add sodium azide to a final concentration of 0.02%. Store in the dark at 4 °C.

Working solution:

Add 0.1 mL of stock solution to 9.9 mL of PBS (pH 7.2). Store in the dark at 4 °C.

Alternate acridine orange hydrochloride solution (Fluka)

Dilute 1:5000 in PBS. Add sodium azide to a final concentration of 0.02%. Store in the dark at 4 °C.

Procedure

- Mix one drop of culture with a small drop of AO on a slide.
- Put on a coverslip.
- Analyse the slide immediately or keep it in the dark.
- Count 5 to 10 fields of vision to estimate the parasitemia (40× objective, 10× ocular).

III:B. Giemsa staining of thick or thin blood films

by **Marianne Lebbad**

Swedish Institute for Infectious Disease Control and Microbiology and Tumor Biology Center (MTC), Karolinska Institutet SE-171 82 Solna, Sweden

e-mail: marianne.lebbad@smi.ki.se

Equipment

light microscope

Materials and reagents

Giemsa stain:

For best and most reproducible results, buy ready-made Giemsa stain from, for example, Sigma–Aldrich. It is quite difficult to prepare a batch that works as well as the commercial one.

Check every new batch of Giemsa stock solution before using it. **Prepare a new working solution for each staining session.**

phosphate buffer, 6.7 mM (pH 7.1):

0.41 g KH_2PO_4

0.65 g $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$

Bring to 1 liter with distilled water.

methanol (reagent grade for analysis, Merck)

Giemsa staining of thick blood films from cultures

- Leave the thick film to dry properly, preferably overnight. In case of hurry, leave it to dry in an incubator at 37 °C for 1 h. Do not fix.
- Prepare a fresh 2% Giemsa solution in phosphate buffer (pH 7.1).
- Put the slide on a staining rack and pour the Giemsa solution on the slide. Leave it to stain for 30 min. (A stronger Giemsa solution will over-stain parasites from culture.)
- Rinse very carefully and gently. Tap water may be used, but do not let the stream run directly on the blood film.
- Leave the slide in an upright position to dry.
- Observe with immersion oil and objective at 100×.

Giemsa staining of thick blood films from patients

- Leave the thick film to dry properly. In case of hurry, leave it to dry in an incubator at 37 °C for 30 min.
- Prepare a fresh 4 to 5% Giemsa solution in phosphate buffer (pH 7.1).
- Put the slide on a staining rack and pour the Giemsa solution on the slide. Do not fix.
- Leave the slide to stain for 20 min.
- Rinse very carefully and gently. Tap water may be used, but do not let the stream run directly on the blood film.
- Leave the slide in an upright position to dry.
- Observe with immersion oil and objective at 100×.

Giemsa staining of thin blood films from patients or cultures

- Air-dry the thin film.

- Fix the film in methanol for about 30 s.
- Prepare a fresh 5 to 10% Giemsa solution in phosphate buffer (pH 7.1).
- Put the slide in a staining jar or on a staining rack and pour the Giemsa solution on the slide.
- Leave it to stain for 20 min.
- Rinse it carefully and thoroughly under running tap water.
- Leave the slide in an upright position to dry.
- Observe the film with immersion oil and objective at 100×.

Reference

Malaria Working Party of The General Haematology Task Force of the British Committee for Standards in Haematology. 1997. The laboratory diagnosis of malaria. Clin Lab Haematol 19(3):165-170.

III:C. Estimation of the percentage of erythrocytes infected with *Plasmodium falciparum* in a thin blood film

by **Marianne Lebbad**

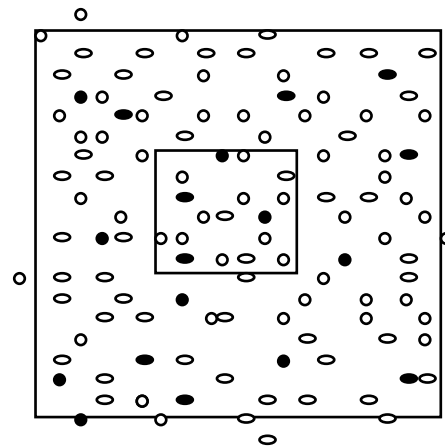
Swedish Institute for Infectious Disease Control and Microbiology and Tumor Biology
Center (MTC), Karolinska Institutet SE-171 82 Solna, Sweden
e-mail: marianne.lebbad@smi.ki.se

Equipment

Use a light microscope with a special ocular (i.e., a reticulocyte ocular) which has a large and a small square where the small square is 10% of the large one (see the figure below, although proportions are not maintained).

Procedure

- Use the 100× objective to view cells under oil immersion.
- Choose an area of a Giemsa-stained thin blood film where the erythrocytes are evenly distributed.
- Count *all* erythrocytes in the small square plus two of the borders.
- Without moving the slide, also count the number of *infected erythrocytes* in the whole area of the big square plus two borders (i.e., including those in the area of the small square).
- Move the slide to “randomly adjacent” fields and continue counting as above (i.e., all erythrocytes of small fields and parasites of accompanying large fields). Continue counting fields until you reach the sum of 100 erythrocytes in the small fields. By extrapolating, the equivalent of 1000 erythrocytes have been examined (regarding parasites).
- Repeat the counting at least twice for a total examination of three different parts of the slide. Take the mean number of infected erythrocytes per 1000 erythrocytes and divide by 10 to get the percent infected erythrocytes.



Please note: If one erythrocyte contains ≥ 2 parasites it is still counted as **one** infected erythrocyte.

Reference

Brecker G, Schneiderman M. 1950. A time saving device for the counting of reticulocytes. Am J Clin Pathol 20:1079-1083.

III:D. Evaluation of parasitemia by lactate dehydrogenase assay

by **Makoto Hirai**^{1,2} and **Ingrid Faye**¹

¹Department of Genetics, Stockholm University, Svante Arrhenius väg 16E, Room E545, SE-106 91 Stockholm, Sweden

²Department of Medical Zoology, Jichi Medical School, 3311-1, Minamikawachi, Tochigi, 329-0498, Japan

e-mail: m.hirai@jichi.ac.jp

Evaluation of parasitemia by lactate dehydrogenase assay is a high throughput method for screening of antimalarial factors.

Equipment

microplate reader
flat-bottomed 96-well plate

Materials and reagents

Malstat reagent (Flow Incorporated)
red blood cells
nitro blue tetrazolium (NBT) stock solution:
100 mg NBT
50 mL distilled water
Dissolve NBT in distilled water. Store in the dark at 4 °C.

phenazine ethosulphate (PES) stock solution:
5 mg of PES
50 mL distilled water
Dissolve PES in distilled water. Store in the dark at –20 °C.

Procedure

- Add 100 µL of Malstat reagent to 96-well microplate.
- Add 20 µL of infected or noninfected red blood cells (hematocrit is 1 to 2%).
- Incubate the plate at room temperature and shake gently several times during incubation to solubilize red blood cells.
- During incubation, mix equal volumes of NBT and PES in the dark and add 20 µL of the mixture to the wells.
- Keep the plate in the dark.
- After 30 to 60 min, check the color development (dark purple).
- Read the plate at 650 nm. Use uninfected red blood cells as reference.
- This assay can be used in 0 to 7% of parasitemia (non-synchronized parasite in 2% of hematocrit).

If you deal with a large number of samples, 20 µL of red blood cells in 96 wells can be stored in 96-well plates at –80 °C before the enzyme assay.

References

Gomez MS, Piper RC, Hunsaker LA, Royer RE, Deck LM, Makler MT, Vander Jagt DL. 1997. Substrate and cofactor specificity and selective inhibition of lactate dehydrogenase from the malarial parasite *P. falciparum*. Mol Biochem Parasitol 90(1):235-246.

Makler MT, Hinrichs DJ. 1993. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. Am J Trop Med Hyg 48(2):205-210

Makler MT, Ries JM, Williams JA, Bancroft JE, Piper RC, Gibbins BL, Hinrichs DJ. 1993. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. Am J Trop Med Hyg 48(6):739-741.

IV. Purification and synchronization of erythrocytic stages

IV:A. Enrichment of knob-infected erythrocytes using gelatine sedimentation

by **Victor Fernandez**

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: victor.fernandez@smi.ki.se

Equipment

immufuge blood centrifuge

Materials and reagents

mature parasite stages

gelatine (Sigma G2625)

RPMI 1640 with HEPES

Procedure

- Make 0.7% gelatine in RPMI 1640 with HEPES.
- Warm and stir the solution at 37 °C until the gelatine is dissolved.
- Filter-sterilize the solution and store it in the refrigerator. Warm it to 37 °C before use.
- Use mature parasite stages. (Ring forms, knobless late stages, and rosettes will sink to the bottom.)
- Wash the culture once using RPMI 1640.
- Resuspend the pellet, approximately 0.2 mL, in 2 mL of 0.7% gelatine solution.
- Incubate the pellet for 1 h at 37 °C.
- Carefully remove the supernatant, which contains the “knobby” parasites, without touching the pellet.
- Centrifuge the supernatant to pellet the parasitized RBC (PRBC) (1 min on high setting in the immufuge blood centrifuge).
- Wash the cells 2 or 3 times using RPMI 1640.
- Subculture the cells using fresh blood and media (see PARASITES, section I).

Reference

Goodyer ID, Johnson J, Eisenthal R, Hayes DJ. 1994. Purification of mature-stage *Plasmodium falciparum* by gelatine flotation. *Ann Trop Med Parasitol* 88(2):209-211.

IV:B. Sorbitol-synchronization of *Plasmodium falciparum*-infected erythrocytes
by **Victor Fernandez**
Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden
e-mail: victor.fernandez@smi.ki.se

Equipment

centrifuge

Materials and reagents

parasite culture of >5% parasitemia
sorbitol
malaria culture medium (MCM)

Procedure

- Take parasites when they are mostly at the ring stage. They must not be later than 10 to 12 h postinvasion when the sorbitol treatment is done.
- Spin down the parasite culture at $600 \times g$ to a pellet (take 4 mL of a culture of >5% parasitemia).
- Add 4 mL of 5% sorbitol (in distilled water) and incubate for 10 min at room temperature. Shake 2 or 3 times.
- Centrifuge the culture at $600 \times g$, and wash it 3 times in malaria culture medium, and dilute it to 5% hematocrit.
- Count parasitemia and subculture as usual.
- Repeat the procedure after one cycle (approximately 48 h).
- To keep the parasites synchronized, the sorbitol treatment must be performed once a week.

Reference

Lambros C, Vanderberg JP. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J Parasitol 65(3):418-420.

IV:C. Enrichment of late-stage infected erythrocytes in 60% Percollby **Victor Fernandez**

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: victor.fernandez@smi.ki.se

Equipment

centrifuge, swing-out rotor, refrigerated

Materials and reagents

mature parasite stages

malaria culture medium (MCM) with the human serum replaced by 10% FBS

60% Percoll solution:

60 mL Percoll

7 mL 10× PBS

33 mL PBS

The solution is enough for 40 tubes of 2.5 mL each, which is enough to enrich 80-mL of culture. Make fresh solutions before each use. Keep all solutions on ice throughout the preparation.

PBS

Procedure

- Wash a mature-stage *Plasmodium falciparum* culture 3 times in MCM with the human serum replaced by 10% FBS and resuspend it to 10% hematocrit in cold MCM with 10% FBS.
- Distribute the culture in centrifuge tubes, 2 mL/tube.
- Using a Pasteur pipette, gently add 2.5 mL of cold 60% Percoll to the bottom of each tube.
- Centrifuge in a swing-out rotor at $1,500 \times g$ and 4 °C for 15 min (2,000 rpm in Sorvall or Beckman centrifuge).
- Withdraw carefully the cells at the interphases, pool them, and wash them 3 times with cold PBS (centrifuging each time for 7 to 8 min at 2,000 rpm). The interphases contain trophozoites and schizonts, while rings and uninfected erythrocytes are found in the pellet.

Reference

Wahlgren M, Berzins K, Perlmann P, Björkman A. 1983. Characterization of the humoral immune response in *Plasmodium falciparum* malaria. I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of microELISA. Clin Exp Immunol 54(1):127-134.

IV:D. Separation of *Plasmodium falciparum* mature stages in Percoll/sorbitol gradientsby **Victor Fernandez**

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: victor.fernandez@smi.ki.se

Equipment

centrifuge, fixed angle rotor (Sorvall SS-34 or Beckman JA-20)
 benchtop centrifuge

Materials and reagents

heparin
 PBS (pH 7.2)
 Percoll
 sorbitol
 RPMI 1640
 distilled water
 malaria culture medium (MCM)
 Corex tubes
 23 G 0.6-mm Ø needle
 tuberculin syringe

Preparation of gradients (for 4 gradients):50 mL of Solution H:

(90% Percoll, 5% sorbitol)
 45 mL Percoll
 5 mL 10× RPMI 1640
 2.5 g sorbitol

30 mL of Solution L:

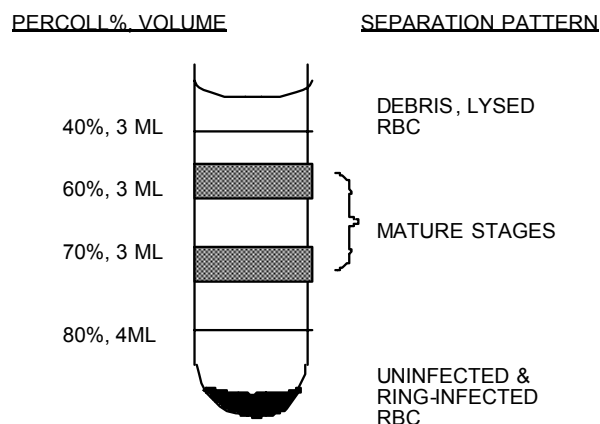
(0% Percoll, 5% sorbitol)
 27 mL distilled water
 3 mL 10× RPMI 1640
 1.5 g sorbitol

Prepare gradients:

<u>Volume</u>	<u>Percoll</u>	<u>Solution L</u>	<u>Solution H</u>
15 mL	40%	8.3 mL	6.7 mL
15 mL	60%	5.0 mL	10.0 mL
15 mL	70%	3.3 mL	11.7 mL
20 mL	80%	2.2 mL	17.8 mL

Procedure

- For each gradient, fill a 15-mL Corex tube as indicated below. Using a syringe, and starting with the lightest solution (40%), carefully layer the next denser layer under the previous one:



Separation of parasite stages

- Wash the PRBC and resuspend them in 1 mL of Solution L.
- Add 100 U/mL of heparin and pass the suspension 5 times through a 23 G 0.6-mm Ø needle using a tuberculin syringe (to disrupt rosettes).
- Very carefully overlay the cell suspension on top of the gradient.
- Centrifuge the gradients in a fixed angle rotor at 10,000 rpm for 30 min at 20 °C. Do not use a brake to decelerate.
- Discard the top of the gradient.
- Collect the parasites (floating as described in the diagram above) in a fresh centrifuge tube, no more than 5 mL/tube in a 10-mL tube. Fill the tube with PBS, mix very gently, and spin at 3,000 rpm for 1 min in a benchtop centrifuge.
- Resuspend the PRBC in MCM (see also PARASITES, section I:A).

Reference

Fernandez V, Treutiger CJ, Nash GB, Wahlgren M. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. Infect Immun 66(6):2969-2975.

IV:E. Obtaining free parasites

by **Denise Mattei**

Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur, 75724 Paris, France

e-mail: dmm@pasteur.fr

Equipment

centrifuge (15-mL tube and microfuge)

Materials and reagents

Plasmion (Fresenius France)

5% solution (w/v) of Gly-Ser (Sigma G3252)

40 mM HEPES

10 mM D-glucose in distilled water

Procedure

- Enrich parasitized red blood cells with Plasmion. Recover them by centrifugation at 2,000 rpm for 2 min. Resuspend the pellet at 37 °C in 1.5 volumes of a 5% solution (w/v) of Gly-Ser made with 40 mM HEPES (pH 7.2) and 10 mM D-glucose in distilled water.
- Incubate at 37 °C for 10 to 30 min.
- Add 5 volumes of culture medium.
- Centrifuge at 1,000 rpm for 3 min. Free parasites are in the supernatant; intact cells are in the pellet. The pellet of intact PRBC can be re-incubated with Gly-Ser.

Reference

Elford B. 1992. Generating viable parasites extra-erythrocytic forms of *P. falciparum*. TDR News 41:11.

IV:F. Obtaining semi-intact cells

by **Denise Mattei**

Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur, 75724 Paris, France

e-mail: dmm@pasteur.fr

In eukaryotic cells this method extracts soluble cytoplasmic components with the membranes retained.

Equipment

refrigerated microfuge

Materials and reagents

Plasmion (Fresenius France)

washing buffer (WB):

10 mM HEPES (pH 7.2)

15 mM KCl

breaking buffer (BrB):

50 mM HEPES (pH 7.2)

90 mM KCl

Procedure

- Enrich parasitized red blood cells with Plasmion. Wash 1 volume of Plasmion (without centrifugation) 3 times in PBS and resuspend it in the same volume of ice cold washing buffer, WB. For example, if you start with 250 μ L of Plasmion, resuspend the pellet in 250 μ L of WB.
- Centrifuge the cells in a refrigerated microfuge at 15,000 rpm for 10 min at 4 °C.
- Wash the pellet once in WB and resuspend it in the original volume of WB.
- Incubate it on ice for 10 min. Recover PRBC by centrifugation at 15,000 rpm for 10 min at 4 °C.
- Resuspend the pellet in the original volume of breaking buffer, BrB.
- Centrifuge as above and resuspend the pellet in one tenth of the original volume of BrB. Semipermeable parasites can be used in immunofluorescence studies or as membrane-enriched extracts on Western blots or immunoprecipitations.

Reference

Beckers CJM, Keller DS, Balch WE. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell* 50(4):523-534.

IV:G. Alanine synchronization of *Plasmodium falciparum*-infected erythrocytes**by Catherine Braun-Breton**

Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut

Pasteur, 75724 Paris, France

e-mail: cbb@pasteur.fr

Only early trophozoites and noninfected erythrocytes will survive this treatment.

Equipment

centrifuge

Materials and reagents

early trophozoites

noninfected erythrocytes

0.3 M alanine

10 mM HEPES (pH 7.5)

culture medium (RPMI 1640 with 10% heat-inactivated human serum)

Procedure

- Prewarm a solution of 0.3 M alanine, 10 mM HEPES (pH 7.5) at 37 °C.
- Spin down the parasite culture to a pellet (5 min at 2,000 × g at 20 °C).
- Resuspend the pellet in 5 volumes of 0.3 M alanine, 10 mM HEPES (pH 7.5).
- Incubate the solution for 3 min at 37 °C.
- Add 10 volumes of culture medium.
- Centrifuge the tube for 5 min at 2,000 × g at 20 °C and resuspend the pellet in culture medium at a 5% hematocrit.

Reference

Braun-Breton C, Rosenberry TL, Pereira da Silva L. 1988. Induction of the proteolytic activity of a membrane protein in *Plasmodium falciparum* by phosphatidyl inositol-specific phospholipase C. Nature 332(6163):457-459.

IV:H. Selection of trophozoites by using magnetic cell sorting (MACS)**by Anna Vogt and Fredrik Pettersson***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: anna.vogt@mtc.ki.se***Equipment**

Vario-MACS magnet (Miltenyi Biotec)
MACS CS-column (Miltenyi Biotec)
3-way stopcock (Miltenyi Biotec)
10-mL syringe
5-mL syringe
0.8-mm needle
0.6-mm needle
50-mL Falcon tubes
microscope
centrifuge

Materials and reagents

4 to 6 mL malaria culture, 5 to 10% parasitemia
PBS
150 mL 2% BSA/PBS
heparin (25000 IU/mL, Lövens)

Preparation

- Connect the 3-way stopcock and the column and place it in the magnet (fig. 1).
- Fill the 10-mL syringe with 2% BSA/PBS and connect it to the left opening of the 3-way stopcock. Open the way between the syringe and the column by closing the way between the syringe and the tap way out (fig. 2). Load the column with 2% BSA/PBS by carefully pushing the connected syringe (fig.3). Fill up to 10 mm above the column matrix. Close the 3-way stopcock totally (fig. 4). Refill the syringe with 2% BSA/PBS and reconnect it to the column. Leave the column for incubation for at least 5 min.
- Preparing the malaria culture: Centrifuge the malaria culture. Resuspend the pellet in 2 mL of 2% BSA/PBS. If using rosetting parasites, add heparin (100 U/mL) and push the culture through a 0.6-mm needle five times. Check in the microscope that all the rosettes are disrupted.
- Cut the top of the cover of a 0.8-mm needle and connect it to the 3-way stopcock (fig. 1). Open the way between the column and the needle and let the excess of loaded 2% BSA/PBS elute. Do not let any air into the column.
- Load the parasites on the top of the column. Open the way between the column and needle. Let the parasites migrate into the column. Close when 1 mm of solution is left. Add some 2% BSA/PBS and let the cells sediment following a quick opening of the tap to let the last cells immigrate. Do not let blood go through the needle. Leave for incubation for 10 min.
- Wash the column by adding 50 mL of 2% BSA/PBS to the top of the column and open between column and the needle. Leave some millimeters of the solution before closing the tap.
- Disconnect the needle from the 3-way stopcock. Disconnect the column from the magnet. Elute the bound material by holding the column upside down over a

Falcon tube and flush through 50 mL of 2% BSA/PBS using the syringe connected to the 3-way stopcock.

- Centrifuge the eluted material at 1,500 rpm for 5 min at room temperature. If using heparin, wash the pellet 5 times in 50 mL of PBS.
- Count the parasitemia.
- The column can be reused immediately. Place the column in the magnet and follow the protocol from the beginning. If reused later, please use the instructions for this procedure delivered together with the columns.

Comments

Approximately 50% of the trophozoites originally found in culture will be an enriched suspension. The following factors have been shown to affect the results:

- The culture should have a parasitemia of 6 to 10%, well synchronized trophozoites.
- The volume of culture used should not be larger than 6 mL or 10^9 cells.
- There should be no (or very few) extracellular parasites in the culture, as they seem to attach better to the matrix.
- A good looking, well-synchronized, culture without extracellular parasites seems to be the most important thing for a good result on the MACS.
- If heparin-free cultures are necessary, rosettes can be disrupted mechanically by passing the culture through the needle without heparin (see above), but make sure all rosettes are disrupted before loading the column.

Reference

Uhlemann AC, Staalsoe T, Klinkert MQ, Hviid L. 2000. Analysis of *Plasmodium falciparum*-infected red blood cells. MACS & more 4(2):7-8. Available from: <http://www.miltenyibiotec.com/index>.

Figures

Fig 1 – Assembly of the MACS column

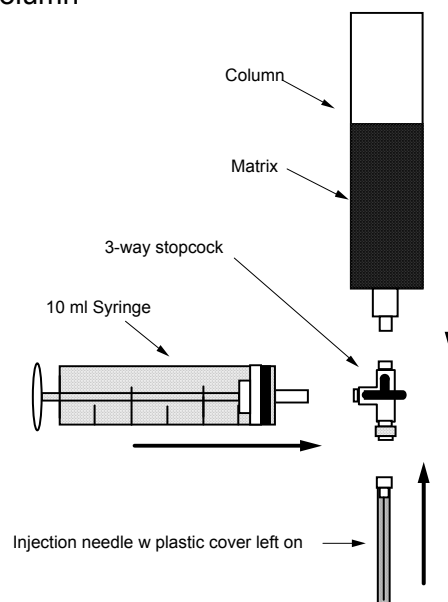


Fig 2.

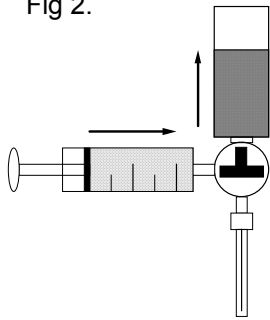


Fig 3.

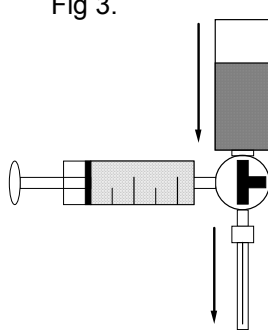
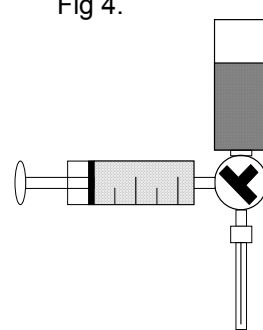


Fig 4.



Data for MACS-column:

Matrix volume	6.3 mL
Reservoir volume	6.5 mL
Maximum capacity total cells	10^9
Maximum capacity retained cells	2×10^8
Rinse volume	60 mL
Wash volume	30 mL
Elution volume	30 mL

IV:I. Isolation of *Plasmodium falciparum*-infected erythrocytes from the placenta

by **James G. Beeson** and **Stephen J. Rogerson**

Department of Medicine, University of Melbourne, Post Office Royal Melbourne Hospital,

Parkville VIC 3050, Australia

e-mail:beeson@unimelb.edu.au

See PARASITES, section VII:A

V. Micromanipulation cloning of *Plasmodium falciparum*

by **Victor Fernandez**

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: victor.fernandez@smi.ki.se

Equipment

inverted microscope (Diaphot 300, Nikon)
micromanipulator system (NT-88, Nikon/NARISHIGE)
micropipette puller (Sutter Instrument, Model P-87)
pipette filling accessories: tubing, needle, syringe, MicroFil (World Precision Instruments)

Materials and reagents

capillaries, Boroglass, outer diameter = 1.0 mm, inner diameter = 0.5 mm (Sutter Instrument)
glass slides
12- or 24-well microplates
0.2- μ m syringe filter
culture flasks, 25 cm²
parasite culture of 5 to 10% parasitemia
malaria culture medium (MCM; see PARASITES, section I:A)
human serum, blood type AB⁺ (HS, see PARASITES, section I:A)
human erythrocytes, blood type O⁺ (see PARASITES, section I:A)
PBS (pH 7.2)
RPMI 1640
acridine orange (see PARASITES, section III:A)
Acrylease (Stratagene)

Preparation

- Pull pipettes in the micropipette puller. We use program 0 (heat: 392, vel: 100, pressure: 599). Each capillary gives two micropipettes. Break the tip with tweezers to get an inside diameter of 3 to 5 μ m. Check in the microscope that the pipette tip edge is blunt.
- Mix MCM with human serum to get 90% MCM/10% HS and filter through a 0.2- μ m syringe filter.
- Fill a pipette with filtered 90% MCM/10% HS. Attach the pipette to a tube connected to a needle/syringe. Put the tip of the pipette in filtered 90% MCM/10% HS and fill the tip by pulling the syringe. Hold for 1 to 2 min until the liquid is above the narrow part of the tip. Detach the syringe. Fill the rest of the pipette by using a MicroFil. Coat the outside of the filled micropipettes by dipping the tip in filtered 90% MCM/10% HS for at least 10 min.
- Attach the micropipette to the micromanipulator system.

V:A. Micromanipulation cloning of parasites

- Add 1 mL of filtered 90% MCM/10% HS to well A1 of a microplate. Make sure that the entire surface of the bottom is covered.
- Incubate the plate for 1 h at room temperature.
- Add 1 or 2 mL of 85% MCM/15% HS to the rest of the wells. Use the smaller volume for 24-well plates and the larger for 12-well plates.

- Add 2 to 8 μL of parasite culture to well A1. Dispense and leave it for 15 min to sediment.
- Pick individual erythrocytes with one parasite each and transfer them to the other wells of the microplate, placing only one parasite-infected erythrocyte in each well. If rosetting parasites are picked, strip off the uninfected erythrocytes and make sure that only one parasite is picked.
- Wash erythrocytes three times with RPMI 1640 and add them to all wells (except A1) to get a hematocrit of 1%; 20 μL to 12-well plates and 10 μL to 24-well plates.
- Culture the cells in candle jars as described below in section C below (see also PARASITES, section I:A).

V:B. Micromanipulation cloning of *Plasmodium falciparum* for single-cell RT-PCR

- Siliconize several slides with Acrylease.
- Put a drop of PBS on one of these slides.
- Exchange the filtered 90% MCM/10% HS in the lower part of the micropipette tip with PBS.
- Put 0.2 to 0.3 mL of PBS on another siliconized slide.
- Add 1 to 2 μL of the parasite culture and mix.
- Let the cells sediment for 5 to 10 min.
- On a siliconized slide, dispense 10 μL of PCR mix (see also MOLECULAR BIOLOGY, section V).
- Transfer 1 infected erythrocyte (with 1 parasite) from the parasite-containing drop to the PCR mix drop.
- Pipette the 10- μL drop containing the infected erythrocyte back into the PCR tube and immediately freeze the tube on dry ice.
- Pick uninfected erythrocytes as controls.

V:C. Expansion of *Plasmodium falciparum* clones

- *Day 0*: Cloning (see sections A and B above).
- *Day 3*: Change the medium. Carefully remove the supernatant and add fresh 85% MCM/15% HS. Add 1 mL to 24-well microplates and 2 mL to the 12-well plates.
- *Day 6*: Change the medium and add 1% hematocrit of washed erythrocytes (10 μL to 24-well plates, 20 μL to 12-well microplates).
- *Day 9*: Change the medium.
- *Day 12*: Change the medium and add 1% hematocrit.
- *Day 15*: Change the medium.
- *Day 17*: Change the medium and add 1% hematocrit.
- *Day 19*: Check the clones. Take a drop from the bottom of the well and put it on a slide. Add a drop of acridine orange (see PARASITES, section III:A) and use the microscope to check for parasites. Examine at least 30 fields. If the parasitemia is $>1\%$, move the clone to a 25- cm^2 culture flask with 90% MCM/10% HS and continue to incubate the culture (see PARASITES, section I:A). Change the medium in wells with very low or no detectable parasitemia.
- *Day 21*: Check wells and/or flasks and record the phenotype of the parasites. Discard empty wells and transfer clones with parasites to culture flasks as above

(see Day 19). When the parasitemia in the flasks is higher than 5% and the parasites are in the ring stage, they can be frozen (see PARASITES, section II).

References

Chen Q, Barragan A, Fernandez V, Sundström A, Schlichtherle M, Sahlén A, Carlson J, Datta S, Wahlgren M. 1998. Identification of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite *P. falciparum*. J Exp Med 187(1):15-23.

Chen Q, Fernandez V, Sundström A, Schlichtherle M, Datta S, Hagblom P, Wahlgren M. 1998. Developmental selection of *var* gene expression in *Plasmodium falciparum*. Nature 394(6691):392-395.

Fernandez V, Treutiger CJ, Nash GB, Wahlgren M. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. Infect Immun 66(6):2969-2975.

VI. Cytoadhesion and rosetting assays

VI:A. Basic cell media

by **Fredrik Pettersson** and **Andreas Hedding**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: fredrik.pettersson@mtc.ki.se

CHO and L cell medium, solution for 500 mL:

5.2 g RPMI 1640 (with glutamine, without NaHCO₃; Gibco)

3.0 g HEPES (Gibco)

0.4 g geneticin

450 mL distilled water

50 mL fetal calf serum

0.5 mL penicillin/streptomycin (Gibco stock concentrations: penicillin, 10,000 IU/mL; streptomycin, 10,000 µg/mL)

Mix components and filter-sterilize; store at 4 °C for up to one month.

Alternative CHO and L cell medium, solution for 500 mL:

450 mL commercial RPMI 1640 (Gibco with 20 mM HEPES, without L-glutamine)

50 mL fetal calf serum

8 mL geneticin (Gibco stock concentration, 50 mg/mL)

50 mL L-glutamine (Gibco stock concentration, 20 mM, 100×)

0.5 mL penicillin/streptomycin (Gibco stock concentrations: penicillin, 10,000 IU/mL; streptomycin, 10,000 µg/mL)

Mix components and store at 4 °C for up to one month.

HUVEC medium, solution for 500 mL:

5.84 g MCDB-131 (Sigma)

3.0 g HEPES (Gibco)

1.0 g NaHCO₃

10 ng/mL (final concentration) epidermal growth factor

1 µg/mL (final concentration) hydrocortisone

0.5 mL penicillin/streptomycin (Gibco stock concentrations: penicillin, 10,000 IU/mL; streptomycin, 10,000 µg/mL)

25 mL fetal calf serum

Mix components and filter-sterilize; store at 4 °C for up to a month.

COS-7 cell medium:

Dulbecco's Modified Eagle Medium (DMEM, Gibco, 41966-029)

10% fetal calf serum

Mix components. Store the medium at 4 °C. Once the serum has been added, use this complete medium within one week.

Binding medium (pH 6.8), solution for 500 mL:

5.2 g RPMI 1640 (with glutamine, without NaHCO₃; Gibco)

3.0 g HEPES (Gibco)

450 mL distilled water

50 mL human serum, inactivated, nonimmune AB⁺ (10%)

Mix components and set pH to 6.8 immediately before use.

Alternatively, RPMI 1640 and HEPES can be mixed, filter-sterilized, and kept at 4 °C for up to a month. Immediately before use, add 10% human serum and set the pH to 6.8.

10× RPMI, solution for 100 mL:

10.43 g RPMI 1640 (with glutamine, without NaHCO₃; Gibco)

6 g HEPES (Gibco)

2 g glucose

100 mL distilled water

Mix components and filter-sterilize. Set pH to 6.8.

VI:B. Thawing melanoma and other cell lines

by **Fredrik Pettersson** and **Andreas Hedding**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: fredrik.pettersson@mtc.ki.se

Equipment

water bath (37 to 40 °C)
centrifuge (Beckman Allegra 6R)
15-mL Falcon tube (BD Labware)
25-cm² culture flask (treated, nonpyrogenic, polystyrene; Corning)
incubator (37 °C, 5% CO₂)

Materials and reagents

appropriate cell medium (see PARASITES, section VI:A)
gelatine (2% gelatine in PBS, warmed for 30 min at 37 °C)

Thawing of cells

- Remove the vial with the frozen cells from the liquid nitrogen container and put it into a water bath at 37 to 40 °C. Thawing should be rapid (40 to 60 s).
- As soon as the ice is melted, remove the vial from the water bath.
- Transfer the contents of the vial to a sterile test tube and centrifuge it at 300 × *g* for 5 min.
- Discard the supernatant.
- Dilute the cell suspension with an appropriate volume of the recommended culture medium.
- Transfer the suspension to a culture flask and incubate it at 37 °C in 5% CO₂. The flask top should be loose. When cultivating HUVEC cells, the bottom of the flask should be coated with warm gelatine before the cells are added.
- In order to expedite the removal of the protective freezing additive (glycerol or dimethyl sulfoxide) from the culture medium, change the culture medium 24 h after thawing unless stated otherwise in the specific instructions for a particular cell line.

VI:C. Freezing of cell linesby **Fredrik Pettersson** and **Andreas Hedding***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: fredrik.pettersson@mtc.ki.se*Equipment

freezing vials (Nunc Cryo Tube Vials; Nalge Nunc International)
15-mL Falcon tube (BD Labware)
Neubauer chamber
microscope (40× lens)
4 °C-refrigerator
–70 °C-freezer
liquid nitrogen tank for long-term storage

Materials and reagents

freezing medium:
7 mL RPMI 1640 medium
1 mL dimethyl sulfoxide (DMSO)
2 mL fetal calf serum (FCS)
EDTA–trypsin solution:
EDTA, 2 mg/mL
trypsin, 2.5 mg/mL
appropriate cell medium (see PARASITES, section VI:A)
trypan blue

Freezing of cells

- Prepare the freezing medium.
- Detach the cells from the cell culture flask with 2 mL of EDTA–trypsin solution and resuspend them in 10 mL of fresh medium (see PARASITES, section VI:D below).
- Count a sample of cells in trypan blue (1:2 dilution) using a Neubauer chamber.
- Centrifuge the sample at 4 °C (300 × g for 5 min) and resuspend the cells with freezing medium at 1×10^6 cells/mL.
- Aliquot the cells into freezing vials and keep them at 4 °C for 2 h.
- Place the vials in –70 °C overnight.
- Place them in liquid nitrogen for long-term storage.

VI:D. Cultivation of CHO, COS, HUVEC, melanoma, and L cells

by **Fredrik Pettersson** and **Andreas Heddini**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: fredrik.pettersson@smi.ki.se

Equipment

centrifuge (Beckman Allegra 6R)
15-mL Falcon tube (BD Labware)
25 cm² culture flask (treated, nonpyrogenic, polystyrene; Corning)
incubator (37 °C, 5% CO₂)

Materials and reagents

RPMI 1640 medium
EDTA–trypsin solution
EDTA, 2 mg/mL
trypsin, 2.5 mg/mL
appropriate medium (see PARASITES, section VI:A)
0.5% gelatine in PBS for HUVEC cells

Passage of cell culture

- Aspirate the old medium from the culture.
- Wash the cells once with RPMI 1640.
- Add 2 mL of EDTA–trypsin.
- Leave the cells for approximately 5 min.
- Shake the flask sharply and check the flask in the inverted microscope to see that the cells have detached.
- Add 10 mL of fresh medium.
- Mix and transfer the suspension to a 15-mL Falcon tube and centrifuge the suspension for 5 min at 300 × *g*.
- Remove the supernatant by aspiration and resuspend the pellet in 1 mL of culture medium.
- Determine the number of cells in a Neubauer chamber.
- Add 5 mL of fresh culture medium to a new 25-cm² flask (for HUVEC cells use a gelatine-coated flask, see note below) and add approximately 10⁵ cells.
- Incubate the cells at 37 °C in 5% CO₂. The flask top should be loose.

Note: Before seeding the HUVECs, coat the culture flasks with 1% gelatine in sterile PBS sterile for 1 h at 37 °C:

- Add 2 to 3 mL of gelatine in PBS to the cell culture flask.
- Incubate the flasks for 30 min at 37 °C.
- Remove the excess gelatine by aspiration and add cells as above.

VI:E. Formaldehyde-fixation of melanoma cells**by Fredrik Pettersson and Andreas Hedding***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: fredrik.pettersson@mtc.ki.se***Equipment**

24-well microplates
cell culture-treated coverslips (Thermanox plastic coverslip; Nalge Nunc International)
incubator (37 °C, 5% CO₂)
inverted microscope (125×)
microscope (1000×)

Materials and reagents

melanoma C32 cells in culture
appropriate medium (see PARASITES, section VI:A)
EDTA–trypsin solution:
 EDTA, 2 mg/mL
 trypsin, 2.5 mg/mL
RPMI 1640 medium
PBS
1% formaldehyde

Fixation of cells

- Place cell culture coverslip with the cell culture-treated side upwards at the bottom of the wells of a 24-well microplate.
- Add 1 mL of appropriate cell cultivation medium.
- Follow the protocol for subcultivation of cells (see PARASITES, section VI:D), but instead of seeding melanoma cells in cell culture bottles, add approximately 50×10^3 cells to each of the wells.
- Leave the melanoma cells to attach to the coverslip in the incubator (37 °C, 5% CO₂) overnight.
- Remove the medium from the melanoma cells and wash twice with PBS.
- Add 0.5 mL of 1% formaldehyde to each well and incubate the cells for 1 h at room temperature.
- Wash them twice with PBS.
- Add PBS and cover the wells; store at 4 °C until use. The maximum storage time is approximately 1 month.

Reference

Schmidt JA, Udeinya IJ, Leech JH, Hay RJ, Aikawa M, Barnwell J, Green I, Miller LH. 1982. *Plasmodium falciparum* malaria. An amelanotic melanoma cell line bears receptors for the knob ligand on infected erythrocytes. J Clin Invest 70(2):379-386.

VI:F. Binding assays to endothelial and melanoma cells**by Fredrik Pettersson and Andreas Hedding***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: fredrik.pettersson@mtc.ki.se***Equipment**

24-well microplates
cell culture-treated coverslips (Thermanox plastic coverslip; Nalge Nunc Int.)
centrifuge (Beckman Allegra 6R)
15-mL Falcon tube (BD Labware)
incubator (37 °C)
glass slides
inverted microscope (125×)
microscope (1000×)

Materials and reagents

melanoma or endothelial cells in culture
malaria culture of 8% parasitemia or higher with a majority of the parasites at trophozoite stage
EDTA–trypsin solution:
 EDTA, 2 mg/mL
 trypsin, 2.5 mg/mL
RPMI 1640
appropriate medium (see PARASITES, section VI:A)
5% Giemsa solution in phosphate buffer (see PARASITES, section III:B)
1% glutaraldehyde in PBS
glycerol
binding medium (see PARASITES, section VI:A).

Procedure

- Place cell culture coverslips with the cell culture-treated side upwards at the bottom of the wells of a 24-well microplate.
- Add 1 mL of appropriate cell culture medium to each well.
- Follow the protocol for subcultivation of cells (see PARASITES, section VI:D), but instead of seeding cells in culture bottles, add approximately 50×10^3 cells to each of the wells with coverslips.
- Leave the cells to attach to the coverslip in the incubator (37 °C, 5% CO₂) overnight. For melanoma cells, please see note at the end of the protocol.
- Inspect cells that have been plated on coverslips; each cell should be separated from other cells for easy reading of binding.
- Centrifuge the malaria culture at $500 \times g$ for 5 min at room temperature.
- Wash the culture 3 times ($500 \times g$) with binding medium and resuspend in binding medium to a hematocrit of about 2%.
- Aspirate medium from the coverslips in the 24-well plate and rinse once with binding medium. Be careful not to let the coverslip with the attached cells dry.
- Add 0.5 mL of malaria culture suspension to each coverslip.
- Incubate the cells at 37 °C for 60 min, gently rocking by hand every 15 min. Do not use a CO₂ incubator.

- Check the binding of malaria-infected erythrocytes to the cells using an inverted microscope.
- Wash away unbound red cells by dipping the coverslip carefully in each of 3 beakers of binding medium or RPMI 1640.
- Fix the coverslips in 1% glutaraldehyde for 60 min or overnight. Alternatively, fix in 1% methanol for 30 min.
- Rinse coverslips with distilled H₂O and stain with 1% Giemsa for 20 to 30 min.
- After staining, rinse the coverslips with water and let them dry.
- Mount the coverslips on glass slides with glycerol.
- Examine the slides under a microscope (oil, 1,000×) and count 300 cells.
- Calculate the average number of infected erythrocytes bound per target cell.

Note: Melanoma cells can be used fresh or fixed with 1% formaldehyde if fixation insensitive receptors are to be studied, such as CD36 (see PARASITES, section VI:E).

Reference

Marsh K, Marsh WM, Brown J, Whittle HC, Greenwood BM. 1988. *Plasmodium falciparum*: the behaviour of clinical isolates in an in vitro model of infected red blood cell sequestration. Exp Parasitol 65(2):202-208.

VI:G. Selection of cytoadherent parasites by passage over C32 melanoma cells, CHO, HUVEC, or other endothelial cells

by **Fredrik Pettersson and Andreas Heddi**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: fredrik.pettersson@mtc.ki.se

Equipment

centrifuge (Beckman Allegra 6R)
incubator (37 °C)
15-mL Falcon tube (BD Labware)
inverted microscope (125×)

Materials and reagents

malaria culture of 8% parasitemia or higher (e.g., enriched) with a majority of the parasites in trophozoite stage
binding medium (see PARASITES, section VI:A above)
25-cm² culture flask covered with melanoma/endothelial cells at approximately 80% confluence
complete malaria culture medium (see PARASITES, section I:A)
human erythrocytes blood type O⁺ (see PARASITES, section I:A)

Selection of cytoadherent parasites

- Use malaria cultures with a parasitemia exceeding 8%; lower parasitemias are not worthwhile, because the binding will be too low. The majority of the parasites should be in the trophozoite stage.
- Transfer the malaria culture to a 15-mL Falcon tube and centrifuge at 500 × g for 5 min at room temperature.
- Wash the culture 3 times (500 × g) in binding medium and resuspend in binding medium at a hematocrit of 2 to 3%.
- Use a 25-cm² culture flask covered with melanoma/endothelial cells at approximately 80% confluence.
- Remove growth medium and wash the melanoma/endothelial cells once with binding medium.
- Overlay the cells with the malaria culture suspension.
- Incubate the cells at 37 °C for 1 to 1.5 h. Do not use a CO₂ incubator.
- Resuspend the cells by gently rocking the flask every 15 min.
- Wash the flask gently 3 times with binding medium to remove unbound erythrocytes.
- Check binding of erythrocytes using an inverted microscope.
- Remove binding medium and add complete malaria culture medium and washed fresh human erythrocytes (see PARASITES, section I:A) at 5% hematocrit to the culture flask.
- Transfer the culture to a new flask the next day (after merozoite reinvasion of RBC).
- Continue culturing (see PARASITES, section I).

Reference

Marsh K, Marsh VM, Brown J, Whittle HC, Greenwood BM. 1988. *Plasmodium falciparum*: the behaviour of clinical isolates in an in vitro model of infected red blood cell sequestration. Exp Parasitol 65(2):202-208.

VI:H. Binding of fluorescent receptors heparin, blood group A, and PECAM-1/CD31 to *Plasmodium falciparum*-infected erythrocytes

by **Fredrik Pettersson** and **Andreas Hedding**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: fredrik.pettersson@mtc.ki.se

Equipment

PCR tubes
centrifuge ($500 \times g$)
microcentrifuge ($500 \times g$)
glass slides
coverslips
UV light microscope (100 $\times$ magnification)

Materials and reagents

malaria culture (with parasites in trophozoite stage)
RPMI 1640
ethidium bromide
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
for heparin:
fluorescein-conjugated heparin (2 mg/mL, Molecular Probes)
for blood group A:
fluorescein-conjugated blood group A (1 mg/mL A_{tri}-PAA-flu, Syntesome)
for CD31:
recombinant PECAM-1/CD31 (800 μ g/mL, R&D Systems)
Alexa Fluor 488 Protein Labeling Kit (Molecular Probes)

Binding of fluorescent receptors

PECAM-1/CD31 is bought unlabelled and must therefore be labelled with a fluorophore before the experiment. Use the Alexa Fluor 488 Protein Labeling Kit to label 400 μ g of CD31 in 0.5 mL of PBS. Follow the protocol provided by Molecular Probes.

- Wash the culture (5% hematocrit) twice in RPMI 1640 ($500 \times g$) and resuspend to original volume.
- Aliquot 30 μ L of washed culture in PCR tubes (0.6 mL). Make one tube for each receptor to be investigated.
- Centrifuge the PCR tubes in a microcentrifuge ($500 \times g$), remove the supernatant, and resuspend the cells to a volume of 26 μ L with RPMI 1640.
- Add 4 μ L of the fluorochrome-conjugated receptor to each PCR tube and mix (regarding CD31 see note below).
- Incubate the cells for 30 min in the dark with resuspension after 15 min.
- Wash the cells 3 times with RPMI 1640 ($500 \times g$) and resuspend them to 30 μ L in RPMI 1640.

When using heparin-FITC or blood group A-FITC, the fluorescence might be very weak. A possibility is to amplify the signal by using Alexa 488 labelled α FITC (antifluorescein, goat IgG fraction, Alexa Fluor 488 conjugate from Molecular Probes) in a second incubation (dilution 1:8, 30 min at room temperature in the dark). After this second incubation, wash the cells 3 times as before and continue as described below.

- Resuspend the cells in 30 μL of RPMI 1640 and mix 10 μL of cells with 0.5 to 1 μL of ethidium bromide (1 $\mu\text{L}/\text{ml}$) on a glass slide. Alternatively, to reduce the amount of background, incubate the cells in the PCR tube with ethidium bromide (1 to 2 μL ; 1 $\mu\text{g}/\text{mL}$) for 5 to 10 s before the last wash.

If fluorescence is weak, ethidium bromide may be skipped altogether as the infected cells can be detected in the microscope by the presence of pigment.

- Add a coverslip and count the fluorescence rate at 100 $\times$ magnification with oil in a UV-light microscope.
- Calculate the fluorescence rate as =
$$\frac{\text{number of fluorescent cells}}{\text{total number of late trophozoites}}$$

References

Heddini A, Chen Q, Obiero J, Kai O, Fernandez V, Marsh K, Muller WA, Wahlgren M. 2001. Binding of *Plasmodium falciparum*-infected erythrocytes to soluble platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): frequent recognition by clinical isolates. Am J Trop Med Hyg 65(1):47-51.

Heddini A, Pettersson F, Kai O, Shafi J, Obiero J, Chen Q, Barragan A, Wahlgren M, Marsh K. 2001. Fresh isolates from children with severe *Plasmodium falciparum* malaria bind to multiple receptors. Infect Immun 69(9):5849-5856.

VI:I. Adhesion of *Plasmodium falciparum*-infected erythrocytes to immobilized receptors

by **James G. Beeson**

Department of Medicine, University of Melbourne, Post Office Royal Melbourne Hospital,
Parkville VIC 3050, Australia

e-mail: beeson@unimelb.edu.au

Equipment

microscope
Class 2 safety cabinet (laminar flow)
centrifuge

Materials and reagents

marking pens, indelible and suitable for plastic
150-mm plastic (polystyrene) tissue culture (petri) dishes
Dako Pen for Immunohistochemistry (Dako Corp) or similar, OR plastic sealing film (e.g., Parafilm, Nescofilm)
liquid paraffin oil
glass coverslips
phosphate-buffered saline (pH 7.2) OR Tris-buffered saline (pH 7.4)
RPMI–HEPES (pH of 6.8 to 7.4) without bicarbonate added
bovine serum albumin
pooled nonimmune human serum OR nonimmune AB serum
2% glutaraldehyde in PBS
Giemsa stain
purified receptors
adhesion medium: RPMI–HEPES (pH of 6.8 to 7.4), plus EITHER 10% human serum (pooled or AB serum from nonexposed donors) OR 1% BSA

Procedure (Beeson et al. 1999)

- On the underside of a plastic petri dish, mark approximately 10-mm diameter circles for the required number of test spots (usually 3 spots for each receptor being tested.) Number or label the circles accordingly.
- Inside the petri dish, on the base, use a Dako pen for immunohistochemistry to copy circles over those drawn above. This creates a circle of plastic that will prevent the parasite suspension being tested (see step 8) from spreading. Alternatively, cut holes in a layer of plastic sealing film using a hole punch and carefully press the plastic film onto the base of the petri dish (see Beeson et al. 1998). Use a blunt instrument (e.g., top of a plastic pen) to press the film on completely, taking care around holes to form a good seal with the plastic dish.
- Place 5 to 10 μ L of each receptor, diluted in PBS or TBS, in the center of each circle.
- Incubate the dishes overnight at 4 °C in a humid box to ensure the receptor spots do not dry out.
- Before performing the adhesion assay, block nonspecific binding by placing a drop or two (50 to 100 μ L) of 1% BSA in PBS over each receptor spot. Incubate for at least 30 min at room temperature.
- Prepare the parasites in a Class 2 cabinet:
 - Spin down parasites from culture or patient sample at low speed.
 - Wash parasite-RBC pellet once with RPMI–HEPES.

- Remove the supernatant and resuspend the pellet in cytoadherence medium (RPMI–HEPES plus either 10% human serum OR 1% BSA) to the required hematocrit, usually 1 to 5%. We typically use mature pigmented trophozoite-infected RBCs at a parasitemia of 1% or higher.
- Wash the plate with either PBS or RPMI–HEPES by adding 25 mL of buffer, agitating, and discarding the liquid. Repeat once.
- Aspirate, using suction, the remaining buffer from each spot and then add 40 μ L of parasite suspension to each well (performed in Class 2 safety cabinet).
- Incubate the cells at 37 °C for 30 min (no agitation).
- Gently wash off unbound cells with RPMI–HEPES (pH of 6.8 to 7.4) or PBS (pH of 7.2 to 7.4).
 - Add 25 mL of buffer carefully to one side of the dish using a pipette. Do not add the buffer directly over receptor spots.
 - Rock the dish gently to remove unbound cells and aspirate the supernatant.
 - Wash the dish 4 to 6 times in total.
- Fix bound cells with 2% glutaraldehyde in PBS for at least 2 h.
- Stain the cells with 10% Giemsa for 10 min, wash off stain with water, and allow to dry. Do not overstain as it becomes difficult to distinguish infected from uninfected RBCs.
- Once they are dry, cover the spots with liquid paraffin oil (or clear vegetable oil) and a glass coverslip.
- Count bound infected-RBCs microscopically, expressing counts as bound parasitized RBCs/mm². Compare adhesion to receptors to that of BSA-coated control spots to account for any nonspecific adhesion.

Comments

Steps 6 – 10 should be performed in a Class 2 safety cabinet.

Purified chondroitin sulfate A (CSA) – We used CSA from bovine trachea at 10 μ g/mL, or from porcine rib at 50 μ g/mL. This works well for most isolates. CSA can be coupled to phosphatidylethanolamine to improve immobilization on plastic (Rogerson et al. 1995).

Different plastic culture dishes can be used in these assays. We generally use Falcon 1058, but others also work.

Various compounds, serum, or antibodies can be tested for inhibitory activity by incubating parasites with the inhibitor for 5 to 45 min prior to step 7 (see Beeson et al. 1998, Reeder et al. 1999).

References

- Beeson JG, Brown GV, Molyneux ME, Mhango C, Dzinjalamala F, Rogerson SJ. 1999. *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. J Infect Dis 180(2):464-472.
- Beeson JG, Chai W, Rogerson SJ, Lawson AM, Brown GV. 1998. Inhibition of binding of malaria-infected erythrocytes by a tetradecasaccharide fraction from chondroitin sulfate A. Infect Immun 66(7):3397-3402.

Beeson JG, Rogerson SJ, Cooke BM, Reeder JC, Chai W, Lawson AM, Molyneux ME, Brown GV. 2000. Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria. *Nat Med* 6(1):86-90 [see comments]

Chai W, Beeson JG, Kogelberg H, Brown GV, Lawson AM. 2001. Inhibition of adhesion of *Plasmodium falciparum*-infected erythrocytes by structurally defined hyaluronic acid dodecasaccharides. *Infect Immun* 69(1):420-425.

Reeder JC, Cowman AF, Davern KM, Beeson JG, Thompson JK, Rogerson SJ, Brown GV. 1999. The adhesion of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate A is mediated by *P. falciparum* erythrocyte membrane protein 1. *Proc Natl Acad Sci USA* 96(9):5198-5202.

Rogerson SJ, Chaiyaroj SC, Ng K, Reeder JC, Brown GV. 1995. Chondroitin sulfate A is a cell surface receptor for *Plasmodium falciparum*-infected erythrocytes. *J Exp Med* 182(1):15-20.

VI:J. Adhesion of *Plasmodium falciparum*-infected erythrocytes to immobilized hyaluronic acid

by **James G. Beeson**

Department of Medicine, University of Melbourne, Post Office Royal Melbourne Hospital,
Parkville VIC 3050, Australia

e-mail: beeson@unimelb.edu.au

Hyaluronic acid (HA) preparations

HA from bovine vitreous humor (Sigma) is isolated by strong anion exchange chromatography and generally has little or no detectable chondroitin sulfates (Chai et al. 2001). Other sources of HA, but not all, also support adhesion. Some preparations of HA may contain significant amounts of copurified chondroitin sulfates.

Immobilization of HA onto plastic

Immobilization is generally effective with PBS or TBS (pH of 7.2 to 7.4) as the coating buffer, and parasite adhesion is enhanced by including calcium in the coating buffer (and in all other steps). HA effectively adsorbs to the surface of Falcon 1058 plastic dishes (tissue culture treated, Becton–Dickinson).

Optimum conditions for adhesion

- Adhesion of parasites to HA is highest at pH 7.4.
- Include calcium in all steps.
- Use mature pigmented trophozoites in assays rather than schizonts.
- Fixation of bound parasites with glutaraldehyde (see PARASITES, section VI:A) may decrease observed adhesion to HA. It may be necessary to count numbers of cells bound to HA prior to fixation.

Materials and reagents

hyaluronate lyase (hyaluronidase) from *Streptomyces hyalurolyticus*

hyaluronic acid isolated from bovine vitreous humor

phosphate-buffered saline

Step-by-step procedure for preparation and use of lyase-treated hyaluronic acid as a control in adhesion assays

- Incubate HA (1 mg/mL) with and without hyaluronate lyase (10 to 20 TRU/mL) in PBS, at 60 °C for 4 h or overnight.
- Stop incubation by boiling HA samples for 10 min at 100 °C. Cool samples and centrifuge them at high speed to remove any insoluble aggregates.
- Make aliquots of treated and untreated HA samples and store them at –20 °C.
- To measure parasite adhesion to HA, dilute lyase-treated and untreated HA samples 1:10 (i.e., a final HA concentration of 100 µg/mL) in PBS and coat plastic surfaces with it as described above (see PARASITES, section VI:A).
- Perform adhesion assay as described above (see PARASITES, section VI:A).
- Interpretation: Adhesion of parasites to nonlyase-treated HA but not to lyase-treated HA indicates specific adhesion to HA.

References

Beeson JG, Rogerson SJ, Cooke BM, Reeder JC, Chai W, Lawson AM, Molyneux ME, Brown GV. 2000. Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria. Nat Med 6(1):86-90. [see comments.].

Chai W, Beeson JG, Kogelberg H, Brown GV, Lawson AM. 2001. Inhibition of adhesion of *Plasmodium falciparum*-infected erythrocytes by structurally defined hyaluronic acid dodecasaccharides. Infect Immun 69(1):420-425.

VI:K. Binding of *Plasmodium falciparum*-infected erythrocytes to placental tissue sections

by **Kirsten Flick** and **Carin Scholander**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet, SE-171 77 Stockholm, Sweden

e-mail: kirsten.flick@mtc.ki.se

See PARASITES, section VII:B

VI:L. Enrichment of rosetting parasites using Ficoll–Isopaque (Pharmacia)
by **Victor Fernandez** and **Antonio Barragan**
Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden
e-mail: victor.fernandez@smi.ki.se

Equipment

immufuge blood centrifuge

Materials and reagents

Ficoll–Isopaque (FIP) (Pharmacia)

RPMI 1640

MCM with 10% heat-inactivated serum

Protocol

- Use 2 mL of culture per tube, mature and rosetting parasites, with a 5 to 10% parasitemia and layer it very carefully on 2 mL of sterile, ice-cold Ficoll–Isopaque (FIP).
- Centrifuge the tube in the immufuge blood centrifuge for 12 s on the high setting at room temperature.
- Quickly suck up the pellet using a Pasteur pipette.
- Wash the pellet 3 times using RPMI 1640.
- Subculture (see PARASITES, section I) using fresh, washed erythrocytes and MCM with 10% heat-inactivated serum.

Reference

Udomsangpetch R, Wåhlin B, Carlson J, Berzins K, Torii M, Aikawa M, Perlmann P, Wahlgren M. 1989. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. J Exp Med 169(5):1835-1840.

VI:M. Reversion of rosettesby **Victor Fernandez** and **Antonio Barragan**

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: victor.fernandez@smi.ki.se

Equipment

UV-light microscope (40×)

Materials and reagents

96-well microtiter plate

Parafilm

acridine orange

Procedure

- Keep the *Plasmodium falciparum* culture under standard conditions with 10% normal serum added to the buffered medium (see PARASITES, section I:A).
- Perform the studies on cultures with 5% hematocrit, 4 to 8% parasitemia at late stage, and >50% rosetting rate.
- Mix an aliquot of 25 to 50 µL of the rosetting *P. falciparum* culture (1:1) with various dilutions (e.g., 1:5) of sera to be tested. Also prepare a control using nonimmune human sera or MCM (see PARASITES, section I:A).
- Incubate the cultures at 37 °C for 60 min in a 96-well microtiter plate. Cover the plate with Parafilm.
- Use two separate aliquots from each well, mix each with a small amount of acridine orange, and mount them on glass slides. Count 25 consecutive fields of vision per slide using a 40× lens and incident UV light (2 slides/well, approximately 50 fields). Count fields of vision diagonally over the slide, from one corner to the other, in order to compensate for a possible uneven distribution of rosettes on the slide. If the cell concentration is low, it may be necessary to count in both diagonal directions.

Comments

Infected erythrocytes (except ring forms) within a rosette and infected erythrocytes not connected to a rosette are scored separately.

Infected erythrocytes that have bound two or more noninfected cells are scored as rosettes. If the rosette contains more than one infected erythrocyte, the cells are scored separately.

Ring forms within a rosette are not counted.

Late-stage (trophozoite and schizont, but not ring forms) infected erythrocytes are scored and added to the total number of late infected erythrocytes.

Since it can be hard to tell the difference between a ring form and an early trophozoite, only parasites with a diameter that covers at least one-third of the diameter of the erythrocyte are counted.

The number of infected erythrocytes in large rosettes (where it can be difficult to determine the exact number of infected cells) are counted as 3.

If it is difficult to determine the number of cells (usually 2 or 3) bound to an infected erythrocyte, the coverslip can be pressed lightly (using a pencil) to induce a slight movement. This is important, because quite often it is found that only 1 erythrocyte is actually bound to the infected cell, when others that may appear to be are not.

Use two controls, one before counting the samples and one after.

Reference

Carlson J, Holmquist G, Taylor DW, Perlmann P, Wahlgren M. 1990. Antibodies to a histidine-rich protein (PfHRP1) disrupt spontaneously formed *Plasmodium falciparum* erythrocyte rosettes. Proc Natl Acad Sci USA 87(7):2511-2515.

VII. Placental malaria

VII:A. Isolation of *Plasmodium falciparum*-infected erythrocytes from the placenta

by **James G. Beeson** and **Stephen J. Rogerson**

Department of Medicine, University of Melbourne, Post Office Royal Melbourne Hospital,
Parkville VIC 3050, Australia

e-mail: beeson@unimelb.edu.au

Equipment

microscope
safety cabinet (laminar flow)
centrifuge
rotating wheel or tube roller
protective eye wear and face mask

Materials and reagents

50-mL plastic tubes
scalpel or scissors, forceps
phosphate buffered saline with 50 mM EDTA

Procedure (Beeson et al. 1999)

- Collect a placenta as soon as possible after delivery, and determine whether it is infected by performing a blood smear. Orient the placenta so the maternal side is facing up. Clean the surface with PBS if necessary.
- From apparently healthy areas of the maternal surface, cut several pieces of placental tissue (approximately 1.5 to 2 cm in each dimension, up to 6 cm³) from different areas using either a scalpel or scissors and forceps. Immediately put cut pieces of placental tissue into 50-mL tubes containing PBS with 50 mM EDTA. Several pieces can be placed into a single tube, but the placental tissue should occupy no more than one-third of the volume. Wear protective eyewear and a face mask during this procedure.
- Mix tubes containing placental tissue on a rotating wheel or tube roller for 1 h at room temperature. To increase yield of placental blood and parasites, placental tissue can be cut into smaller pieces or finely chopped after 30 min, and the incubation then continued for a further 30 min. Doing this does not appear to significantly influence the parasitemia or parasite phenotypes of the preparation.
- Stop incubation, then compress the placental tissue with a blunt instrument to further remove any blood and parasites. Let tubes stand for one minute to allow tissue pieces and debris to settle.
- Collect the supernatant, then centrifuge and wash the pellet 3 times with PBS.
- Prepare a thin smear and determine the parasitemia of the preparation.
- Leukocytes can be depleted by passing a suspension of the placental blood over a cellulose column or over Percoll density gradients.
- If necessary, the parasitemia can be enriched by passage over Percoll density gradients or by other methods, as described elsewhere.

References

Beeson JG, Brown GV, Molyneux ME, Mhango C, Dzinjalama F, Rogerson SJ. 1999. *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. J Infect Dis 180(2):464-472.

Beeson JG, Rogerson SJ, Cooke BM, Reeder JC, Chai W, Lawson AM, Molyneux ME, Brown GV. 2000. Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria. Nat Med. 6(1):86-90.

VII:B. Binding of *Plasmodium falciparum*-infected erythrocytes to placental tissue sections

by **Kirsten Flick** and **Carin Scholander**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: kirsten.flick@mtc.ki.se

Equipment

cryostat at -20°C

Materials and reagents

binding medium:

5.2 g RPMI powder

3.0 g HEPES

500 mL distilled H_2O

liquid nitrogen

methanol

distilled H_2O

hyaluronidase: 10 $\mu\text{g}/\text{mL}$ enzyme in PBS (pH 7.2)

chondroitinase: 0.5 units/mL in Tris-HCl (pH 8.6)

heparinase: 0.5 units/mL in PBS

PBS

slides with 8 wells of 8 mm or 10 wells of 6 mm each (Novakemi AB)

coverslips

50-mL Falcon tubes

Placenta sections:

- Cut fresh placental tissue into small cubes ($\approx 1 \times 1 \text{ cm}$) and freeze immediately in liquid nitrogen. Transfer them later to -70°C for storage.
- Adapt the placental tissue cubes to -20°C for 1 h before use.
- Cut 5- μm thick sections with a cryostat at -20°C .
- Transfer the sections to 8- or 10- well slides (see below).
- Keep the slides with sections at -70°C . The sections should not be older than 1 week when used in experiments.
- For use, remove a slide from -70°C , transfer it immediately into cold PBS, and keep it in PBS until use.

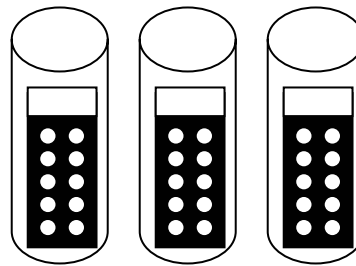


Preparation of PRBC:

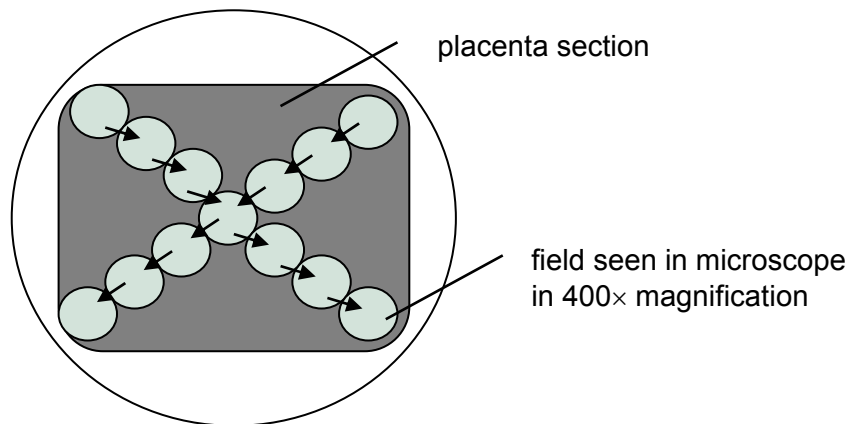
- Enrich the infected cells for late trophozoites in a Percoll-gradient or by MACS purification.
- Keep the cells as packed cells on ice after purification.

Binding of PRBC to placenta sections:

- Resuspend 1 to 2 μL of packed cells in 23 to 24 μL of binding medium.
- Add those 25 μL to one well containing a placental section, forming a drop on top of the section.
- Incubate the section for 1 h at 37 °C.
- Tip off the drop carefully.
- Wash the section 3 times for 5 min in binding medium: Use three 50-mL Falcon tubes with 40 mL of binding medium each; let the slide stand in each for 5 min.
- Fix the sections on a slide for 15 s in methanol.
- Let them air dry.
- Stain the sections with 5% Giemsa for 30 min (overlay sections with a drop of Giemsa stain).
- Wash away the Giemsa stain with distilled H_2O .
- Put a coverslip over the sections.

Counting of bound PRBC in the microscope:

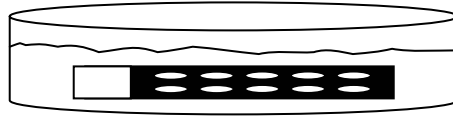
- Count from upper left corner to lower right corner of the section, field next to field at 400 $\times$ magnification.
- Repeat from upper right corner to lower left corner.
- Determine bound PRBC/field, calculate to PRBC/ mm^2 .

Binding of PRBC to placenta sections under influence of inhibitor molecules:

- Incubate the placenta sections with 25 μL of binding medium or PBS containing the inhibitor for 1 h at 37 °C.
- Wash the sections for 3 min with PBS in a petri dish placed on a slow-moving shaker (see below).
- AND/OR: Resuspend PRBC in binding medium containing the inhibitor and incubate them for 1 h at 37 °C.

Perform the binding assay as described above.

Include at least 2 wells with a positive control (i.e., with no inhibitor present) on each slide to control the quality of the experiment.



Enzyme treatment of placenta sections:

- Use a volume of 25 μ L on each section:
 - hyaluronidase: 10 μ g/mL of enzyme in PBS (pH 7.2); incubate at 37 °C for 45 min.
 - chondroitinase: 0.5 units/mL in Tris–HCl (pH 8.6); incubate at 37 °C for 10 to 30 min.
 - heparinase: 0.5 units/mL in PBS, incubate at 37 °C for 30 min.
- Wash the sections for 3 min with PBS in petri dishes.
- Include at least 2 wells with a positive control (i.e., no enzyme treatment) on each slide to control the quality of the experiment.

Perform the binding assay as described above.

Reference

Flick K, Scholander C, Chen Q, Fernandez V, Pouvelle B, Gysin J, Wahlgren M. 2001. Role of nonimmune IgG bound to PfEMP1 in placental malaria. *Science* 293(5537): 2098-2100.

VIII. Detection of antibodies to the infected erythrocyte surface

VIII:A. Reversion of rosettes

by **Victor Fernandez** and **Antonio Barragan**

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: victor.fernandez@smi.ki.se

See PARASITES, section VI:M

VIII:B. Agglutination assay using purified trophozoite-infected erythrocytes.

Measurement of antibodies to *Plasmodium falciparum* variant antigens on the surface of infected erythrocytes

by **James G Beeson** and **Emily J. Mann**

Department of Medicine, University of Melbourne, Post Office Royal Melbourne Hospital, Parkville VIC 3050, Australia

e-mail: beeson@unimelb.edu.au, e-mann@pgrad.unimelb.edu.au

Materials and reagents

phosphate-buffered saline (PBS)
microscope slide
glass coverslips
mounting medium (e.g., Depex)
96-well microtitre plates (Eppendorf tubes [1.5 mL], or similar)
Giemsa staining (see PARASITES, section III:B)
human serum
methanol

Procedure

- Isolate trophozoite-infected RBCs (not schizonts) from culture using Percoll density gradients, gelatin, or other methods, described elsewhere.
- After harvesting parasites, carefully and gently wash 3 times with warm PBS.
- Make a smear, stain it with Giemsa, and examine the parasite preparation. Check for intact parasite-infected RBCs. Aim for at least 50% parasitemia (higher is better).
- Incubate the parasites with human serum diluted 1:10 for 45 min on a rotating wheel at room temperature.
This is best done by putting 2.5- μ L aliquots of each test serum into individual wells of a 96-well microtitre plate (or using Eppendorf tubes). Add 22.5 μ L of parasite suspension (at a 2% hematocrit) to each well containing test serum. Always include a negative control serum.
Parasites harvested from 25 mL of culture (5 to 10% parasitemia, 3% hematocrit) can usually be resuspended in 0.5 to 1.0 mL of PBS.
- Following incubation, pipette out 10 μ L of the suspension and spread it gently with a plastic pipette tip onto a glass slide, making a circle of about 1.5-cm diameter. Make 2 smears for each sample on the same or separate slides. [Alternatively, to examine cells as 'wet preps', place a coverslip over a 10- μ L drop (do not smear out) and examine by microscopy (see below)].
- Allow the smear to dry for 5 min.
- Fix the smears in fresh methanol.

- Stain the smears for 5 to 10 min with 10% Giemsa, but do not over-stain. Once the smears are dry, mount coverslips using a permanent mounting medium (e.g., Depex). Slides can then be stored (protected from light) for long periods and examined or re-examined at any time.
- Examine both smears for each sample by light microscopy.
 - Initially scan for agglutinates using a 10× objective.
 - Examine any agglutinates at 40×.
 - Consider as positive only those agglutinates of 5 infected cells or more.
 - Record the percentage of parasitised RBCs in agglutinates and the size of the largest agglutinate observed.

Comments

Count only agglutinates of parasitised RBCs. Don't count agglutinates of parasites that have broken out of the RBC membrane (which frequently and easily agglutinate). Make sure you can see an intact RBC membrane around cells in agglutinates.

Watch out for agglutinates of cells with debris. Agglutinated cells must be in contact with each other.

Do not count linear agglutinates. Cells lined up in a row are rarely true agglutinates. Find an area where cells are evenly distributed.

VIII:C. Serum micro-agglutination of infected erythrocytesby **Antonio Barragan**

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: antonio.barragan@smi.ki.se

EquipmentUV light microscope
rotatorMaterials and reagentsRPMI 1640/HEPES/sodium bicarbonate/ gentamycin solution (MCM; see
PARASITES, section I:A)
acridine orange (see PARASITES, section III:A)
polystyrene round-bottomed tubesProcedure

- Culture parasitized red blood cells (PRBC) to the late trophozoite and/or early schizont stage and wash the cells 3 times in MCM; resuspend the PRBC at 20% hematocrit.
- Dispense aliquots of 25 μ L in prelabelled polystyrene round-bottomed tubes.
- Add 25 μ L of serum to the parasite suspension (1:2 dilution). For a 1:5 dilution serum test, add prediluted serum at 1:2.5 in MCM.
- Incubate the PRBC and serum mixture at 37 °C for 1 h with constant rotation.
- Mount an aliquot from each polystyrene tube, mix with a small amount of acridine orange on a glass slide, and count 50 consecutive fields of vision diagonally using a 40 $\times$ lens and incident UV-light microscopy.
- Always use negative and positive control sera along with the test sera.

Interpretation

- Score the assay as negative when no agglutinates of 4 or more PRBC are detected in the examined slide.
- Use the following semiquantitative scoring scale for agglutination analysis:
 - (–) for no agglutinate of 4 or more PRBC
 - (1+) for 1 to 5 agglutinates of 4 to 10 PRBC
 - (2+) for >5 agglutinates of 4 to 10 PRBC or 1 to 5 agglutinates of 11 to 20 PRBC
 - (3+) for >5 agglutinates of 11 to 20 PRBC or 1 to 5 agglutinates of >20 PRBC
 - (4+) for >5 agglutinates of >20 PRBC
 - (UA) for unspecific agglutination
- When using rosetting strains, note the presence or absence of rosettes.

References

Aguiar JC, Albrecht GR, Cegielski P, Greenwood BM, Jensen JB, Lallinger G, Martinez A, McGregor IA, Minjas JN, Neequaye J, et al. 1992. Agglutination of *Plasmodium falciparum*-infected erythrocytes from east and west African isolates by human sera from distant geographic regions. Am J Trop Med Hyg 47(5):621-632.

Barragan A, Kremsner PG, Weiss W, Wahlgren M, Carlson J. 1998. Age-related buildup of humoral immunity against epitopes for rosette formation and agglutination in African areas of malaria endemicity. *Infect Immun* 66(10):4783-4787.

Marsh K, Howard RJ. 1986. Antigens induced on erythrocytes by *P. falciparum*: expression of diverse and conserved determinants. *Science* 231(4734):150-153.

VIII:D. Flow cytometry detection of surface antigens on fresh, unfixed red blood cells infected with *Plasmodium falciparum*

by **Helene Jouin**

Unité d'immunologie moléculaire des parasites, CNRS URA 1960, Institut Pasteur, 75724 Paris, France

e-mail: hajouin@pasteur.fr

The indirect immunofluorescence labelling is performed as usual on living cells in suspension, and parasitized erythrocytes are selected by gating the fluorescence intensity of either Hoechst 33 342 or thiazole orange (TO) dyes, which reflect the erythrocyte content of parasitic DNA or RNA. TO allows identification of only mature stages of the parasite. Nevertheless, since we have not observed surface immunostaining of the young stages of the parasite when using Hoechst, TO is a good reagent because it allows performing the flow cytometric analysis with a single laser analyser.

Materials and reagents

Hoechst 33 342 (Sigma)

thiazole orange (TO) (Retic-COUNT, BD Immunocytometry Systems)

FACSFlow solution (BD Immunocytometry Systems) containing 2% fetal calf serum

General procedures for DNA or RNA staining, surface immunofluorescence staining, and flow cytometry analysis

When Hoechst is used, the DNA staining is performed first, followed by surface immunofluorescence staining (using FITC or phycoerythrin conjugated second antibody). It is necessary to use two lasers because of the differing excitatory wavelengths of the two fluorochromes.

When TO is used, surface immunofluorescence staining is performed before the RNA staining. The samples can be analysed on a single laser (488 nm). Since TO fluorescence is read using the FITC channel, FITC conjugates cannot be employed for surface immunofluorescence.

Intraerythrocytic parasite DNA staining with Hoechst 33 342 (before surface immunofluorescence staining)

- Dissolve Hoechst 33 342 at 1 mg/mL of distilled water.
- Add the solution directly to the culture medium at a final concentration of 20 µg/mL.
- Incubate the culture for 30 min at 37 °C in the dark.
- Wash the culture in PBS and then perform the immunofluorescence staining.

Intraerythrocytic parasite DNA staining with TO (after immunofluorescence staining)

- Resuspend the washed immunofluorescent stained parasitized red blood cells directly in 1 mL of a solution of TO.
- Keep in the dark for 30 min prior to flow cytometric analysis.

Surface immunofluorescence staining

- Resuspend a pellet of 5×10^6 parasitized red blood cells in 100 µL of immune serum diluted 1:50 in FACSFlow solution containing 2% fetal calf serum.

- Incubate the pellet at room temperature for 30 min.
- Wash it 3 times with FACSFlow-FCS.
- Resuspend the pellet in the appropriate fluorescent-conjugated antibody.
- Incubate it at room temperature for 30 min.
- Wash it twice with FACSFlow-FCS.
- Resuspend it in 1 mL of FACSFlow.

Flow cytometric analysis

Parasitized red blood cells that have been stained with Hoechst 33 342 must be analysed on a cell sorter equipped with two lasers: a UV laser tuned to 320 nm for the excitation of the Hoechst dye and a second laser tuned to 488 nm for the excitation of FITC and phycoerythrin.

Parasitized red blood cells which have been stained with TO and phycoerythrin conjugates can be analysed on a standard flow cytometer equipped with only one laser tuned to 488 nm. The TO emission is detected in the FITC channel. The color compensation between TO and phycoerythrin fluorescences is set up using a control sample (without surface immunofluorescence staining).

Erythrocytes are gated on the basis of their forward scatter and side scatter signals using logarithmic scales. Parasitized erythrocytes are gated on the basis of their positive staining with Hoechst 33 342 or TO. All fluorescence parameters are recorded with logarithmic amplification. Process list mode data from 10,000 gated cells.

Reference

Jouin H, Goguet de la Salmoniere YO, Behr C, Huyin Qan Dat M, Michel JC, Sarthou JL, Pereira da Silva L, Dubois P. 1995. Flow cytometry detection of surface antigens on fresh, unfixed red blood cells infected by *Plasmodium falciparum*. J Immunol Methods 179(1):1-12.

VIII:E. Analysis of plasma antibodies to variant surface antigens by flow cytometry
by **Trine Staalsoe**

Centre for Medical Parasitology, Department of Infectious Diseases M7641,
Rigshospitalet, Copenhagen, Denmark
e-mail: tscmp@rh.dk

Equipment

2-color flow cytometer

Materials and reagents

PBS + 2% FCS (PBS2)

Plasmodium falciparum late-stage infected erythrocytes purified by magnetic separation (see PARASITES, section IV:H) at 2×10^6 erythrocytes/mL in PBS2

ethidium bromide (0.1 mg/mL in PBS)

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

human plasma or serum

goat anti-human IgG antibody (DAKO A473)

FITC-conjugated rabbit anti-goat Ig antibody (DAKO F250)

Procedure

Antibody labelling

- Add ethidium bromide to the erythrocyte suspension (20 μ L/mL).
- Mix 100 μ L of ethidium bromide-labelled erythrocyte suspension with 5 μ L of test plasma/serum and incubate the tube for 30 min at 5 °C.
- Wash the suspension twice in 3 mL of PBS2 and resuspend the sample in 100 μ L of PBS2.
- Mix the erythrocyte suspension with anti-human antibody (diluted 1:250) and incubate it as above.
- Wash the suspension twice in 3 mL of PBS2 and resuspend the sample in 100 μ L of PBS2.
- Mix the erythrocyte suspension with anti-goat antibody (diluted 1:25), and incubate it as above.
- Wash the suspension once in 3 mL of PBS2 and resuspend the sample in 200 μ L of PBS2.
- After antibody labelling, samples may be kept at 5 °C overnight before analysis.

Data acquisition by flow cytometry

- Adjust flow cytometer settings to achieve a clear separation of uninfected (ethidium bromide-negative) and infected (ethidium bromide-positive) erythrocytes in an FSC/ethidium bromide dot plot (see Fig. 1 in Staalsoe et al. 1999).
- Acquire and store data on FCS, SSC, FL1 (FITC), and FL2 (ethidium bromide).

Data analysis

- Flow cytometry data files (FCS format) can be analysed in any of the many software packages available. We usually use either the freeware WinMDI program (which is good for generating overlays and other graphics for publication) or WinList (which is a very powerful analysis program with wonderful

possibilities for creation of macros that make a breeze of the analysis of the hundreds of files you will often need).

- Set a gate around late-stage infected erythrocytes in an FSC/ethidium bromide diagram plot (similar to R3 in Fig. 1D in Staalsoe et al. 1999).
- Use the FITC fluorescence of the cells within this gate to quantify antibody recognition of variant surface antigens. This can be expressed either as the mean/median fluorescence or as the percentage of cells with FITC fluorescence above that of a control sample incubated without plasma/serum or with control plasma/serum (viz., Fig. 2 in Staalsoe et al. 1999).

Reference

Staalsoe T, Giha HA, Dodoo D, Theander TG, Hviid L. 1999. Detection of antibodies to variant antigens on *Plasmodium falciparum*-infected erythrocytes by flow cytometry. Cytometry 35(4):329-336.

IX. Immunoglobulin- or serum protein-binding to infected erythrocytes

IX:A. Stripping erythrocytes of bound serum proteins and reformation of rosettes

by **Fredrik Pettersson** and **Carl Johan Treutiger**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: fredrik.pettersson@mtc.ki.se

Equipment

sterile test tubes
centrifuge ($500 \times g$)
96-well plates
microscope ($400\times$)

Materials and reagents

500 μ L of a rosetting malaria culture (5% hematocrit, rosetting phenotype with a majority of the parasites in trophozoite stage)

PBS (pH 7.2)

heparin, 25000 IU/mL (Leo Pharma)

sodium citrate

RPMI 1640–HEPES medium (50 mL):

0.52 g RPMI 1640 medium (with glutamine, without NaHCO_3 ; Gibco)

0.3 g HEPES (Gibco)

45 mL distilled water

Mix and set pH to 6.8.

Disruption of rosettes with heparin

Since the rosettes of every parasite strain differ in their heparin-sensitivity, you will have to titrate the necessary heparin-concentration (e.g., FCR3S1.2: 100 U/mL).

- Transfer 500 μ L of culture to an appropriate test tube and add the necessary amount of heparin.
- Incubate the culture for 15 min before the next step.

Treatment with sodium citrate to disrupt heparin resistant rosettes

- Centrifuge the culture at $500 \times g$ and remove the supernatant.
- Add 500 μ L of PBS with 100 mM sodium citrate (pH 7.4) to the pellet and incubate it for 5 min.
- Wash the pellet twice with PBS; centrifuge it each time ($500 \times g$) and remove the PBS.
- Add 0.25 mL of RPMI 1640–HEPES to the pelleted malaria culture (the hematocrit will be 10%).
You may include 10% H-Albumin (Sigma), but this can interfere or block cell interactions and the binding to solid surfaces.

Rosette reformation

- Prepare the test substance (e.g., sera or proteins) in $2\times$ concentration of above RPMI 1640 medium.

- Mix 25 μ L of the washed culture (10% hematocrit; as above) with 25 μ L of the test substance in a 96-well plate. The final hematocrit will be 5% (H-Albumin concentration, if included, will be 5% as well).
- Incubation probably works as well at room temperature as it does at 37 °C for 15 min.
- Read the rosetting rate (=reformed rosettes) using acridine orange (see PARASITES, section III:A)

Reference

Treutiger CJ, Scholander C, Carlson J, McAdam KP, Raynes JG, Falksveden L, Wahlgren M. 1999. Rouleaux-forming serum proteins are involved in the rosetting of *Plasmodium falciparum*-infected erythrocytes. Exp Parasitol 93(4):215-224.

IX:B. Detection of serum proteins on the surface of *Plasmodium*-infected erythrocytesby **Fredrik Pettersson** and **Carl Johan Treutiger***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: fredrik.pettersson@mtc.ki.se*Equipment

centrifuge ($500 \times g$)
 appropriate test tubes for centrifuge
 microfuge tubes
 glass slides
 coverslips
 UV-light microscope ($100\times$)

Materials and reagents

malaria cultures (5 to 10% trophozoites)
 antibody against serum protein to be detected (e.g., αIgM)
 fluorochrome conjugated secondary antibody
 RPMI 1640
 ethidium bromide ($1 \mu\text{g/mL}$)

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

Immunofluorescence

- The cultures to be tested should have a parasitemia of 5 to 10% with a majority of parasites in trophozoite stages.
- Transfer the culture to appropriate test tubes and wash ($500 \times g$) it twice in RPMI 1640. Add RPMI 1640 to the initial volume.
- Dilute antibody to double final concentration of desired dilution in RPMI 1640. (A dilution factor of two will be introduced during the assay because of cell suspension.)
- Aliquot $50 \mu\text{L}$ of each dilution of antibody in small tubes (e.g., microfuge tubes) and add $50 \mu\text{L}$ of resuspended culture.
- Mix by shaking the tubes gently and incubate them for 30 min in the dark at room temperature with careful resuspension after 15 min.
- Shake the tube gently after 15 min.
- Wash the cells 3 times ($500 \times g$) in 0.5 mL of RPMI 1640.
- Remove the supernatant carefully and add $100 \mu\text{L}$ of secondary antibody diluted to the final concentration. Mix gently.
- Incubate the cells for 30 min in the dark with careful resuspension after 15 min and wash them as before.
- Resuspend the cells in $40 \mu\text{L}$ of RPMI 1640 and mix $10 \mu\text{L}$ of cells with 0.5 to $1 \mu\text{L}$ of ethidium bromide ($1 \mu\text{g/mL}$) on a glass slide.
- Add a coverslip and count the fluorescence rate at $100\times$ magnification with oil in a UV-light microscope.
- Calculate the fluorescence rate as =
$$\frac{\text{number of fluorescent cells}}{\text{total number of late trophozoites}}$$

Note: To reduce the amount of background the cells can be incubated with ethidium bromide (1 to 2 μ L; 1 μ g/mL) for 5 to 10 s before the last wash instead of being mixed with ethidium bromide on the slide.

Note: Count all nonfluorescent cells with the corresponding size of the parasite as the parasites in the fluorescent cells. This is important to achieve the correct fluorescence rate, which could otherwise be too high.

Reference

Scholander C, Treutiger CJ, Hultenby K, Wahlgren M. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nat Med* 2(2):204-208.

IX:C. Binding and eluting antibodies from the *Plasmodium*-infected RBC surface

by **Fredrik Pettersson** and **Martha Schlichtherle**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: fredrik.pettersson@mtc.ki.se

Equipment

immufuge blood centrifuge
round-bottomed test tubes (e.g., 5-mL snap-cap tubes)
microfuge tubes
high speed microcentrifuge with cooling system
high quality pH paper

Materials and reagents

P. falciparum culture with a parasitemia of >8%. (If rosetting is of interest, the rosetting rate should be >50%.)

serum or antibody solution to be investigated

RPMI 1640

PBS (optional)

acridine orange

Solution A:

0.2 M glycine / 0.2 M NaCl:

3.75 g glycine

2.9 g NaCl

Bring to 250 mL in distilled water.

Solution B: (0.2 M HCl)

Mix 1.64 mL of concentrated HCl into 98.4 mL of distilled water.

Note: Mix acid into water, never vice versa with concentrated acids.

Glycine–HCl elution solution (pH 3):

Mix 5 mL of solution A and 1.3 mL of solution B.

Adjust to pH 3 using a high quality pH paper or pH meter.

Make fresh for each day of elution.

Neutralization solution: (2 M Tris-base)

9.69 g Tris-base

Bring to 40 mL; pH will be ~11.

Regarding disruption of rosettes and stripping of serum bound proteins, see Materials and reagents in PARASITES, section IX:A..

If using Percoll/sorbitol gradients to separate trophozoites for binding antibodies to the parasite, see Materials and reagents in PARASITES, section IV:D.

Procedure

- Use at least 1 mL of culture where the parasitemia is >8 to 10%. (If rosetting is of interest, the rate should be >50%; see PARASITES, sections VI:L, M.)

- If purified trophozoites are desired for the antibody binding, use Percoll/sorbitol gradients as in PARASITES, section IV:D. Have a large prep ready with either 12 small bottles (25-cm²) or 3 large ones (75-cm²) of culture for 8 Percoll gradients. Follow the protocol for gradients in section IV:D, then resuspend purified cells in 0.5 to 1 mL of RPMI 1640 before the addition of serum/antibodies, followed by the protocol below from the incubation/elution step onward.
- If using the culture straight as it is without trophozoite purification, place the culture in a round-bottomed tube holding a few milliliters and fitting an appropriate blood centrifuge (e.g., a 5-mL snap-cap tube in an immufuge blood centrifuge). Disrupt rosettes using heparin and a syringe, then strip cells of serum-bound proteins (see PARASITES, section IX:A above).
- After the sodium citrate treatment of cells, wash them once with PBS and then with RPMI 1640 (see section IX:A above).
- Add an equal volume of serum or antibody solution to the cell pellet as the starting volume of culture (i.e., mg/mL concentrations of antibody).
- Gently mix and slant the tube so that the blood solution almost reaches the surface of the tube. Carefully roll to mix the settling RBCs every 15 min or so. Incubate the tube for 2 to 3 h at room temperature.
- While the tube is incubating, prepare the glycine-HCl elution solution. At the end of the incubation, check that RBCs look normal through acridine orange vital staining (see PARASITES, section III:A).
- Spin down the cells (500 × g), remove the supernatant, and wash the cells twice with PBS.
- Elute bound antibodies by adding glycine-HCl elution solution to cells in the amount of the starting culture volume. Incubate them for 1 to 1.5 min only! (It is possible that the bound antibodies can be eluted with less elution buffer for increased antibody concentration; this would have to be tested empirically.)
- Quickly transfer the cells to 2 microfuge tubes and centrifuge them at 8,000 to 10,000 rpm in a chilled centrifuge for 1 min.
- Taking care not to touch the cell pellets, remove the supernatant with the eluted antibodies and pool them into one tube.
- To each milliliter of elution, add 26 to 30 µL of 2 M Tris-base. Mix carefully and use 4 µL to spot on a high quality filter paper. Check the pH. Add more Tris-base, 1 µL at a time, until the pH reaches 7.0 to 7.2. Check the pH after each addition. If the eluate becomes too basic, lower the pH by adding 10 to 20 µL of 0.2 M HCl (= solution B).
- Remove the precipitate that may form by spinning the eluate for a minute in the blood centrifuge.
- If you wish, you may dialyze the eluate overnight against a few changes of PBS in the cold, stirring it slowly.

For storage, you may add sodium azide to 0.02% and a “tiny tad” of pure human serum albumin (e.g., Sigma A3782) if this does not interfere with your subsequent assays.

You can use these eluted antibodies in surface immunofluorescence (see PARASITES, section IX:B above) or in standard Western blotting. Typically, 1:2 or 1:4 dilution of the eluted antibodies (from serum) would be visible in surface immunofluorescence, and 1:10 to 1:20 dilutions in Westerns.

IX:D. Enrichment of immunoglobulin binding parasites using Dynabeads

by **Fredrik Pettersson** and **Andreas Hedding**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: fredrik.pettersson@mtc.ki.se

Equipment

Magnetic Particle Concentrator (MPC-1, Dynal)
haemocytometer
cryotubes
rotator for incubating cryotubes
25-cm² cell culture flask
centrifuge for washing RBC (immunofuge II, Baxter)
microscope (400×)

Materials and reagents

uncoated Dynabeads M-450 Tosylactivated (90 mg/mL, Dynal)
appropriate antibodies for coating the Dynabeads
0.1 M borate
PBS 0.1% HSA (50 mL PBS + 50 mg albumine, filter-sterilized)
PBS 10% HSA (50 mL PBS + 5 g albumine, filter-sterilized)
acridine orange
RPMI 1640
MCM without serum (see PARASITES, section I:A)
MCM with 10% serum (see PARASITES, section I:A)

Enrichment of the parasites

- Coat uncoated Dynabeads M-450 Tosylactivated with antibodies using the protocol provided by Dynal.
- Count the parasitemia of the *Plasmodium falciparum* culture and determine the stages of the parasites using acridine orange (see PARASITES, section III:A). It should have a parasitemia of approximately 10% and the parasites should be in trophozoite stages.
- Determine the number of erythrocytes per milliliter using a haemocytometer. Calculate the number of target cells (=infected RBCs) by multiplying the number of cells with the parasitemia. One or two milliliters of parasite culture (approximately 5×10^7 target cells) is usually enough to achieve a good parasitemia after enrichment.
- Calculate the volume of Dynabeads to have 6 Dynabeads per target cell in the solution.
- Wash the Dynabeads 3 times with 3 mL of RPMI 1640 using a 3.6-mL cryotube and a Magnetic Particle Concentrator.
- Wash the culture 3 times ($500 \times g$) with MCM without serum (see PARASITES, section I:A).
- Resuspend the erythrocytes in approximately 2 mL of RPMI 1640 and add the RBCs to the Dynabeads in the cryotube.
- Immediately incubate the tube on a rotator at room temperature for 1.5 to 2.0 h, during which time the trophozoites will attach to the coated Dynabeads.

- After the incubation, before the beads/erythrocytes have time to settle, use the Magnetic Particle Concentrator to remove the medium while the PRBC attached to magnetic beads are bound to the MPC-1.
- Wash the beads/erythrocytes very gently, 1 or 2 times with RPMI 1640 using the Magnetic Particle Concentrator.
- Resuspend the beads/erythrocytes in MCM with 10% serum (see PARASITES, section I:A) in a 25-cm² cell culture flask at a hematocrit of 5% (i.e., 3.8 mL of MCM with serum, 0.2 mL of packed erythrocytes, and the Dynabead-bound *Plasmodium falciparum* infected erythrocytes in a 25-cm² flask).

During the next day, remove the beads from the culture using the Magnetic Particle Concentrator. The parasitized erythrocytes should by then have infected the free erythrocytes in the culture. Continue culturing the remaining erythrocyte mixture as described in PARASITES, section I.

Reference

Heddi A, Treutiger CJ, Wahlgren M. 1998. Enrichment of immunoglobulin binding *Plasmodium falciparum*-infected erythrocytes using anti-immunoglobulin-coated magnetic beads. Am J Trop Med Hyg 59(5):663-666.

X. Fixation protocols for transmission electron microscopy

X:A. Fixation of tissue samples

by **Anne von Euler**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: anne.von.euler@mtc.ki.se

Please note: Use only glutaraldehyde of high purity, “EM-grade”. Purchase only small amounts at any one time, store them at -20°C , and use them as soon as possible. Also, use only paraformaldehyde powder. It is important to dissolve the paraformaldehyde in alkaline buffer before adjusting the cacodylate buffer to neutral pH.

Please note: **ALL CHEMICALS used in this protocol ARE EXTREMELY TOXIC.**

Solutions

For the solutions below:

- Dissolve paraformaldehyde in double-distilled water in a FUME HOOD.
- Stir and heat the solution until it reaches 60°C .
- Add 1.0 N NaOH dropwise (1 to 2 drops) until the milky white solution turns clear.
- Cool this solution before adding cacodylate and glutaraldehyde.
- Adjust the pH with 1.0 M NaOH or 2 M HCl.
- Store solutions at 4°C in dark bottles and use within 1 month or before a white precipitate appears.

Fixation buffer, 100 mL:

0.1 M sodium cacodylate (dimethylarsinic acid sodium salt trihydrate for synthesis), 1% glutaraldehyde, 1% paraformaldehyde (pH 7.4):

1 g paraformaldehyde (> 95% pure)

2.2 g sodium cacodylate

1.4 mL 70% glutaraldehyde (1.96 mL if 50% GA stock), EM-grade

For adjusting the pH to 7.4, use 2 M HCl. Adjust volume to 100 mL using double-distilled water

Fixation storage buffer, 100 mL:

0.1 M sodium cacodylate, 0.1% glutaraldehyde, 0.1% paraformaldehyde (pH 7.4):

0.1 g paraformaldehyde (> 95% pure)

2.2 g sodium cacodylate

0.14 mL 70% glutaraldehyde (0.2 mL if 50% GA stock), EM-grade

For adjusting the pH to 7.4, use 2 M HCl. Adjust volume to 100 mL using double-distilled water.

Procedure

- Cut pieces of tissue to a maximum size of 0.5 cm^3 .
- Immediately immerse the tissue cube in fixation buffer and leave it for 30 min.
- Aspirate the fixation buffer and fill the tube with fixation storage buffer. Fill the tube to the top to avoid contact with oxygen which will cause formalin crystals in the sample.
- Close the tube carefully and seal it with Parafilm.
- Store the samples at 4°C . When being transported, the samples do not need to be at 4°C if that is not possible.

Reference

Scholander C, Treutiger CJ, Hultenby K, Wahlgren M. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nat Med.* 2(2):204-208.

X:B. Embedding samples for electron microscopy

by Anne von Euler

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: anne.von.euler@mtc.ki.se

Equipment

Oven placed in a fume hood or connected to special ventilation

Materials and reagents

Durcupan (Fluka)

acetone

First Durcupan solution:

1 part Durcupan

2 parts acetone

Second Durcupan solution:

2 parts Durcupan

1 part acetone

0.1 M sodium cacodylate (EM-buffer)

1% OsO₄ in 0.1 M EM-buffer

All ethanol used should be diluted from 99.5% ethanol.

99.5% ethanol

70% ethanol

50% ethanol

95% ethanol plus 2% uranyl acetate

Cell samples should be in small tubes, which allow good mixing. Use short glass Pasteur pipettes and break off the thin tip when handling the Durcupan. Use a special waste container for the Durcupan, one that allows the acetone to evaporate in the fume hood and the Durcupan to polymerize and detoxify.

Use 0.1 M sodium cacodylate as a buffer since phosphate buffer can produce precipitates in some samples. It is referred to as EM-buffer in the protocol.

Please note: **ALL CHEMICALS used in this protocol ARE EXTREMELY TOXIC.** Work in a fume hood at all times and use proper waste containers for the chemicals.

Day 1:

- Aspirate the supernatant and rinse the cells 3 times for 5 min each in 0.1 M EM-buffer.
- To give contrast, add enough 1% OsO₄ in 0.1 M EM-buffer to cover the sample.
- Incubate the sample for 45 min at 37 °C or for 1.5 h at room temperature. The OsO₄ evaporates easily, so seal the tubes properly.
- Aspirate the OsO₄ and discard it in a proper waste container for OsO₄. Rinse the sample 3 times for 5 min each in EM-buffer.
- For dehydration, add each of the reagents listed below, one at a time for 5 min each at room temperature. Aspirate and discard the supernatant after each 5-min interval. All ethanol used should be diluted from 99.5% ethanol.
 - 50% ethanol
 - 70% ethanol

95% ethanol plus 2% uranyl acetate

99.5% ethanol

acetone

- Add the first Durcupan solution and leave the sample for 2 to 5 h at room temperature.
- Aspirate the Durcupan/acetone solution and add the second Durcupan solution and leave the sample overnight at room temperature.
- *Day 2:*
- Aspirate the Durcupan/acetone solution, add pure Durcupan, and leave the sample overnight at room temperature.
- *Day 3-4 or 3-5:*
- Change Durcupan daily until polymerization.
- Put a little label in the Durcupan, for identification, quite close to the sample itself.
- Polymerize the samples in a 50 °C oven for at least 20 to 30 h. The polymerization can be done over a weekend. The oven should be placed in a fume hood or connected to special ventilation.

Reference

Scholander C, Treutiger CJ, Hultenby K, Wahlgren M. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nat. Med.* 2(2):204-208.

X:C. Fixation of erythrocytes for immuno-electron microscopy

by **Martha Schlichtherle** and **Anne von Euler**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: anne.von.euler@mtc.ki.se

Equipment

centrifuge

Materials and reagents

parasite culture with desired parasitemia

RPMI 1640

0.1 M phosphate buffer (pH 7.4)

Fixation solution, 100 mL (1% paraformaldehyde, 0.2% glutaraldehyde, 0.1 M phosphate buffer (pH 7.4):

Prepare phosphate buffer stocks (Na_2HPO_4 and NaH_2PO_4), then mix for 0.2 M (pH 7.4) according to Maniatis or "Current Protocols in Molecular Biology".

Mix 1.0 g paraformaldehyde in 50 mL of double-distilled water. In a FUME HOOD, stir and heat the solution until it reaches 60 °C, add 1.0 N NaOH dropwise (1 to 2 drops) until the milky white solution turns clear.

50 mL 0.2 M phosphate buffer

0.4 mL glutaraldehyde (50% stock)

This fixing solution lasts for 2 months when stored at 4 °C.

Procedure

- Grow a parasite culture until desired parasitemia is reached.
- Cool RPMI 1640, 0.1 M phosphate buffer, and fixing solution to 4 °C.
- Wash cells in RPMI 1640 twice and once in cold 0.1 M phosphate buffer (pH 7.4).
- Fix samples in cold fixation solution for 10 min at 4 °C.
- Wash the sample three times in cold 0.1 M phosphate buffer. If using microfuge tubes, spins at 2,500 rpm for 1 min at 4 °C suffice.
- If shipping samples, fill the tubes completely with 0.1 M phosphate buffer, cap them well, and ship them on ice by express.
- If shipping antibodies, add sodium azide for a final concentration of 0.01% and send them on ice by express.

References

Method developed by Dr. Masamichi Aikawa.

Ausubel FM, et al., eds. Current protocols in molecular biology. New York: Wiley-Interscience; 1988-1999.

Quakyi IA, Matsumoto Y, Carter R, Udomsangpetch R, Sjölander A, Berzins K, Perlmann P, Aikawa M, Miller LH. 1989. Movement of a falciparum malaria protein through the erythrocyte cytoplasm to the erythrocyte membrane is associated with lysis of the erythrocyte and release of gametes. Infect Immun 57(3):833-839.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

XI. In vitro reinvasion and growth inhibition assays

XI:A. In vitro reinvasion and growth inhibition assay by microscopy of erythrocyte monolayers

by **Birgitta Wahlin-Flyg**

Octapharma AB, SE-112 75 Stockholm, Sweden

e-mail: birgitta.wahlin-flyg@octapharma.se

Equipment

centrifuge
UV-microscope

Materials and reagents

human type O+ RBCs
parasitized red blood cells (PRBC)
TCM:
 HEPES-buffered (20 mM) RPMI 1640 medium
 10% normal human serum
 2 mM glutamine
 gentamycin (25 µg/mL)
 0.2% NaHCO₃
Tris-buffered Hanks' solution (TH)
0.06 M bicarbonate buffer (pH 9.6)
1% glutaraldehyde in PBS
acridine orange (10 µg/mL)
96-well flat-bottomed tissue culture plates
conical tubes
8-well multitest slides
coverslips

Procedure

- Using normal human type O⁺ RBCs, dilute parasitized red blood cells (PRBC) from *Plasmodium falciparum* cultures infected with late trophozoites and schizonts to a parasitemia of approximately 1% and adjust the hematocrit to 2% with TCM.
- Set up quadruplicate tests in 96-well flat-bottomed tissue culture plates. Use 100 µL of parasite culture mixed with 100 µL of TCM or various dilutions of antibodies.
- Incubate the plate at 37 °C for 18 to 20 h in a candle jar (see PARASITES, section I:A).
- Transfer the quadruplicate tests separately to 4 conical tubes, wash them by centrifugation 2 times with 1 mL of Tris-buffered Hanks' solution (TH), followed by a dilution to 1% hematocrit, by adding 150 µL of TH.
- Make monolayers in duplicates from each tube on 8-well multitest slides that have been pretreated for 30 min with 0.06 M bicarbonate buffer (pH 9.6).
- After 30 min, wash the slides in TH, fix them by two treatments for 10 s each with 1% glutaraldehyde in PBS, wash them with distilled water, and air-dry them.
- Store the slides at room temperature until they are analysed in the UV-microscope.

Analysis of percent parasitemia by UV-microscopy

- To analyse the parasites, stain by adding one drop of acridine orange (10 µg/mL) per well for a few seconds, then wash the slides with distilled water and mount a coverslip.
- Screen 25 microscope fields per well (200 visual fields/slide). Since the number of RBCs/microscope field has been estimated to be 200/field, the percent parasitemia can be calculated from a total of 40,000 RBCs/culture:
- Calculate the percent parasitemia as:

$$\frac{(\text{percent parasitemia in control} - \text{percent parasitemia in sample}) \times 100}{\text{per cent parasitemia in control}}$$

Reference

Udomsangpetch R, Wåhlin B, Carlson J, Berzins K, Torii M, Aikawa M, Perlmann P, Wahlgren M. 1989. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. J Exp Med 169(5):1835-1840.

XI:B. In vitro reinvasion and growth inhibition assay by flow cytometric measurement of parasitemia using propidium iodide (PI) staining

by **Alice Nyakeriga**

*Immunology, Stockholm University, Stockholm, Sweden, and
Moi University FHS, Department of Immunology, Eldoret, Kenya
e-mail: anyakeriga@hotmail.com*

This method allows for quantification of parasitemia and for quantification of parasites in different stages of differentiation according to DNA content. Many similar protocols have been published using different intercalating dyes such as acridine orange, thiazole orange, hydroethidine, or YOYO-1. Each of them has advantages and disadvantages. Below we describe our variant that we find easy and reliable. Care should be taken to distinguish nucleated cells from parasitized erythrocytes when analyzing material containing nucleated cells. The protocol can be extended by additional labeling of cells using FITC-, PE-, and APC-labeled antibodies.

Equipment

centrifuge
flow cytometer
FACScan (Becton-Dickinson)
FACScalibur (Becton-Dickinson)

Materials and reagents

PBS
PBS containing 0.025% (v/v) glutaraldehyde
PBS containing 0.01% saponin
PBS containing 2% FCS
propidium iodide (PI)

Procedure

- Take 1×10^6 to 2×10^6 red blood cells (RBCs) from a culture or a drop of blood from an infected individual collected into heparinized isotonic buffer (e.g., PBS or 0.9% sodium chloride).
- Wash the cells in PBS twice.
- To fix the cells, resuspend them in 1 mL of PBS containing 0.025% (v/v) glutaraldehyde, and incubate them at room temperature for 20 min. Alternatively, incubate the cells at 4 °C for 30 to 45 min. Cells may be stored in the fixative for several weeks before proceeding.
- Centrifuge the cells for 5 min at $450 \times g$. Aspirate the supernatant followed by two washes in PBS.
- Permeabilization:
 - Resuspend the cells in 0.5 mL of PBS containing 0.01% saponin.
 - Incubate them at room temperature for 5 min.
- Wash the cells in PBS twice at $450 \times g$ for 5 min.
- Resuspend the cells in 0.5 to 1.0 mL of FACS buffer. (We use PBS containing 2% FCS.).
- Staining with propidium iodide: To the above cell suspension, add PI to a final concentration of 10 µg/mL. Incubate the cells at 37 °C for 1 to 2 h.
- Analyze the cells in a flow cytometer with a 488-nm laser for excitation, and detect emission from intercalating PI in the 670-nm long pass filter (red FL3

channel). We use the FACScan and the FACScalibur from BD and detect emission in FL3.

References

Barkan D, Ginsburg H, Golenser J. 2000. Optimisation of flow cytometric measurement of parasitemia in *Plasmodium*-infected mice. *Int J Parasitol* 30(5):649-653.

Makler MT, Lee LG, Recktenwald D. 1987. Thiazole orange: a new dye for *Plasmodium* species analysis. *Cytometry* 8(6):568-570.

Pattanapanyasat K, Udomsangpetch R, Webster HK. 1993. Two-color flow cytometric analysis of intraerythrocytic malaria parasite DNA and surface membrane-associated antigen in erythrocytes infected with *Plasmodium falciparum*. *Cytometry* 14(4):449-454.

Whaun JM, Rittershaus C, Ip SH. 1983. Rapid identification and detection of parasitised human red cells by automated flow cytometry. *Cytometry* 4(2):117-122.

Wyatt CR, Goff W, Davis WC. 1991. A flow cytometric method for assessing viability of intraerythrocytic hemoparasites. *J Immunol Methods* 140(1):23-30.

XI:C. ³H-hypoxanthine incorporation assay for the study of growth inhibition by drugs
by **Berit Aydin Schmidt**
Swedish Institute for Infectious Control, SE-171 82 Solna, Sweden
e-mail: berit.schmidt@smi.ki.se

Apparatus

speed vac (Savant Plus SC 110 A)
Harvester (Tomtec Mach III)
scintillation counter (1450 Microbeta Liquid)
Candle jar
Bag sealer

Equipment

96-well cell culture plates, flat-bottomed with lid (Costar)
Eppendorf tubes
Falcon tubes, 14-mL
pipettes, tips 5- to 200- μ L
dispenser (Eppendorf Multipette plus)
Eppendorf Combitip Plus for 0.5 mL
filter papers (Printed Filtermat A Wallac)
sample bags (Wallac)
gloves
racks

Materials and reagents

parasite strain (FCR 3 S1) in culture (unsynchronized)
complete culture medium: RPMI 1640 with 10% human serum from blood group AB+ = MCM (see PARASITES, section I:A)
human erythrocytes, blood type O⁺
test compounds dissolved in DMSO (in conc. 5 mM)
DMSO (dimethyl sulfoxide)
acridine orange (see PARASITES, section III:A)
Beta-plate scintillation fluid
chloroquine diphosphate, in two concentrations, 40 nM and 640 nM, as a control drug
stock solution for control drug 1 (64×10^{-5} M chloroquine diphosphate (MW: 515.9 g/mol):
33 mg chloroquine diphosphate
100 mL distilled water.
Prepare the stock solution. Store at 4 °C in the dark in a plastic flask.
(The drug adheres to glass.)

working solution for control drug 1:

Prepare the working solutions of control drug 1 by diluting the stock solution by 1:100 in RPMI 1640 (containing 1% DMSO); 64×10^{-7} M = control drug 1

working solution for control drug 2:

Make two-fold dilutions of the working solution for control drug 1 in 4 steps; 4×10^{-7} M = control drug 2

8-³H-hypoxanthine (Amersham), 1 mCi/mL in 50% ethanol

Aliquot 100 μ L (in 100- μ L Eppendorf tubes) and store at -20°C .

Prepare the working solution by evaporating the alcohol in a speed vac.

Resuspend the isotope in 5 mL of RPMI 1640 (i.e., 20 μ L isotope/mL of RPMI 1640).

Prepare a new working solution for each assay.

Preparation

Day 1

Work in a sterile hood and use sterile tips and tubes.

- Dilute the test compounds (25 per microplate)
 - A: 1/100 5 μ L in 0.5 mL RPMI
 - B: 1/1000 50 μ L of A in 0.5 mL RPMI
 Final concentrations will be: A: 5 μ M, B: 0.5 μ M
- Prepare a dilution of washed, uninfected human type O⁺ erythrocytes at 2% hematocrit in MCM as background.
Example: 20 μ L O⁺ blood in 1 mL MCM. Add 1 μ L DMSO (to achieve the same concentration as in the test samples).
- Estimate the parasitemia (X%) in the malaria culture and prepare a parasite suspension with 0.2% parasitemia and 2% hematocrit. A 20-mL suspension is enough for 3 plates. (Calculate the amount of parasite culture to add for a 0.2% parasitemia in 20 mL of MCM, add 0.4 mL of O⁺ blood for 2% hematocrit.)

	B 2	B 3	2 A	2 B	8 A	8 B	14A	14B	20A	20B	
	C 2	C 3	3 A	3 B	9 A	9 B	15A	15B	21A	21B	
	D 2	D 3	4 A	4 B	10A	10B	16A	16B	22A	22B	
	E 2	E 3	5 A	5 B	11A	11B	17A	17B	23A	23B	
	F 2	F 3	6 A	6 B	12A	12B	18A	18B	24A	24B	
	1 A	1 B	7 A	7 B	13A	13B	19A	19B	25A	25B	

- Fill all wells around the border of the 96-well plate with a few drops of RPMI.
- Add 10 μ L of control drug I to each of the wells E2 and E3.
- Add 10 μ L of control drug II to each of the wells F2 and F3.
- Add 10 μ L of the test compounds in dilutions A and B above to each of the wells 1A, 1B (G2, G3) to 25 A, 25B (G10, G11), resulting in 25 compounds in two dilutions per plate.
- Add 90 μ L of the uninfected erythrocytes to each of the wells B2 and B3 for background.
- Add 90 μ L of the parasite suspension to each of the wells C2, C3, D2, and D3 for controls.
- Add 90 μ L of the parasite suspension to each of the remaining wells with control drugs and test compounds.

- Continue in the same way with the next plate.
- Note the date, starting time, and number of the plate on the lids.
- Put the plates in a candle jar and incubate them at 37 °C for 24 h. Use a thin layer of sterile water on the bottom of the jar.

Day 2

- Add 25 µL of the ³H-hypoxanthine working solution to each well (0.5 µL/well) and continue the incubation for another 18 h.

Day 3

- Harvest the cells to labeled filter papers in a cell harvester and dry them in an incubator for 1 h.
- Put the filters in sample bags and add 3.2 mL of scintillation fluid.
- Seal the bags and see that the fluid is evenly distributed over the filters.
- Read the cpm in a MicroBeta counter.
- Estimate the inhibition:

$$\% \text{ inhibition} = 100 - \frac{[(\text{cpm in test well} - \text{cpm in B2,3}) \times 100]}{\text{cpm in C2,3} - \text{cpm in B2,3}}$$

Control drug 1 inhibits the growth of FCR3 S1 by >90%

Control drug 2 inhibits the growth of FCR3 S1 by 10 to 30%

References

Chulay JD, Haynes JD, Diggs CL. 1983. *Plasmodium falciparum*: Assessment of in vitro growth by ³H-hypoxanthine incorporation. Exp Parasitol 55(1):138-146.

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob Agents Chemother 16(6):710-718.

XI:D. Plasmodium falciparum hemozoin formation assay**by Mats Wahlgren***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: mats.wahlgren@mtc.ki.se***Equipment**

high speed Immufuge blood centrifuge
centrifuge with Beckman JA-20 rotor
vortex
spectrophotometer (560-nm)

Materials and reagents

malaria culture
4-mL Falcon tubes (BD Labware, 2058)
1.5-mL microcentrifuge tube
5% sorbitol, prepared in distilled water and filtered through a 0.22- μ m filter
MCM with 10% serum
Triton X-100
distilled water
N NaOH
pyridine
2.5 mM potassium ferricyanide, $K_3Fe(CN)_6$
sodium hydrosulfite, $Na_2O_4S_2$
ferriprotoporphyrin IX chloride (Fluka or Sigma) (optional)

Procedure

Grow *Plasmodium falciparum* in MCM medium containing 10% human type AB⁺ serum and 5% hematocrit (type O⁺ blood) according to the candle-jar method of Trager and Jensen (see PARASITES, section I:A).

Synchronization of parasites (see also PARASITES, sections IV:B, G)

- Perform 1 cycle (or more if necessary) of synchronization by sorbitol lysis. Choose a culture with a majority of rings.
- Spin the 4-mL culture for 1 min at “high” speed in an immufuge blood centrifuge using 4-mL Falcon tubes.
- Add 4 mL of 5% sorbitol to the pellet.
- Mix the culture thoroughly by inversion and let it stand for 10 min.
- Spin again for 1 min.
- Wash the pellet 3 times in MCM with 10% serum.
- Return the cells to a culture flask.
- Wait until next ring stage period. Repeat the synchronization 2 more times if necessary.

Growth of parasites with drugs

- Use cultures at the ring stage (12 to 18 h) and a parasitemia of ~5%.
- Change the cultures to fresh MCM (with 10% serum) before adding the drugs.
- Add test drugs to a final concentration of 1 or 10 μ g/mL (or concentrations in between).

- Controls:
 - untreated PRBC culture (as background)
 - PRBC culture treated with chloroquine, or other anti-malarial drugs (e.g., Fansidar), at 1 and 10 $\mu\text{g/mL}$
- Mix by inversion and split the culture into flasks or wells in a cell culture plate.
- Grow a 4-mL culture for each time point in a 25-cm² cell culture flask or a 2.5-mL culture for each time point in a 6-well plate.
- Allow the culture to mature for ~30 h with or without an inhibitory drug.

Pyridine-hemochrome method for the measurement of haem incorporation in hemozoin

- Harvest the culture at various time points spread over ~30 h and measure hemozoin content; e.g., use cultures at: 0 h, 18 h, 20 h, 25 h, and 30 h.
- Transfer contents of the flasks/wells to 10-mL centrifuge tubes which hold for the centrifugation below.
- Add Triton X-100 to a final concentration of 1% (4 mL of a 2% Triton X-100 solution to a 4-mL culture). Leave a small volume of culture for 5% Giemsa staining (see also PARASITES, section III:B) and for counting the parasitemia.
- Spin at 4 °C and 13,000 rpm for 45 min (Beckman JA-20 rotor).
- Discard the supernatant and save the pellet.
- Resuspend the pellet with 1 mL of distilled water and transfer it to a 1.5-mL microcentrifuge tube.
- Spin it at 4 °C and 13,000 rpm for 15 min in a microcentrifuge. This step can be repeated to further wash away any free haem.
- Discard the supernatant and save the pellet.

Do the following steps in a VENTILATED HOOD since pyridine and potassium ferricyanide are VERY TOXIC. Collect waste in a flask, label it appropriately, and send it as organic waste.

- If one wishes to set up a standard curve for the assay, one can use hemin which is ferriprotoporphyrin IX chloride (Fluka or Sigma). Make standard working solutions in distilled water from a stock solution of hemin prepared in DMSO, as hemin is not directly soluble in water. Then take 520 μL of these prepared hemin standard solutions and treat them exactly as the dissolved hemozoin pellet below.
- Add 520 μL of distilled water to the tube with the culture hemozoin pellet, followed by 62 μL of N NaOH and 123 μL of pyridine (for a 4-mL sample). Use half of these volumes for a 2.5-mL sample. Vortex to dissolve the pellet. Add the same volumes of NaOH and pyridine to the hemin standard tubes.
- Split the mixture into equal parts in 2 microcentrifuge tubes. Add 20 μL (or 10 μL for a 2.5-mL sample) of 2.5 mM potassium ferricyanide to one tube to oxidize haem. Add “a pinch” of sodium hydrosulfite to the other tube to reduce haem. Mix by inversion.
- Measure the absorbance in a spectrophotometer at the wavelength of 560 nm.
- Calculate the relative amount of hemozoin at each time point as well as for the hemin standards:

$$\Delta \quad \text{OD}_{560} = \text{OD}_{560} (\text{reduced sample}) - \text{OD}_{560} (\text{oxidized sample})$$

- Plot hemozoin content in the culture versus time and compare relative to culture without drugs and to culture with chloroquine and/or Fansidar, as well as to the standards.

References

Asawamahsakda W, Ittarat I, Chang C-C, McElroy P, Meshnick SR. 1994. Effects of antimalarials and protease inhibitors on plasmodial hemozoin production. *Mol Biochem Parasitol* 67(2):183-191.

Francis SE, Gluzman IY, Oksman A, Knickerbocker A, Mueller R, Bryant ML, Sherman DR, Russell DG, Goldberg DE. 1994. Molecular characterization and inhibition of a *Plasmodium falciparum* aspartic hemoglobinase. *EMBO J* 13(2):306-317.

Slater AFG, Cerami A. 1992. Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. *Nature* 355(6356):167-169

MOSQUITOES AND PARASITES

I. Rearing of *Anopheles stephensi* mosquitoes

by **Martin Looker** and **Andrew W. Taylor-Robinson**

School of Biology, University of Leeds, Leeds LS2 9JT, UK

e-mail: m.locker@leeds.ac.uk, a.w.taylor-robinson@leeds.ac.uk

See MOSQUITOES AND PARASITES, section II:C

II. Culturing of sexual, oocyst, and sporozoite stages

II:A. Plasmodium falciparum gametocyte culture, purification, and gametogenesis

by **Mrinal Kanti Bhattacharyya** and **Nirbhay Kumar**

Kumar Lab, Johns Hopkins Malaria Research Institute, Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, USA

e-mail: nkumar@jhsph.edu

Equipment

incubator (37 °C)

centrifuge

culture flasks (25-cm², 75-cm², and 150-cm², Corning, Canted neck flasks)

Materials and reagents

human RBC washed in incomplete medium

incomplete medium

RPMI 1640

25 mM HEPES

0.37 mM hypoxanthine (pH 7.4)

complete medium

incomplete medium (above) containing 10% heat-inactivated pooled normal human serum

29 mM NaHCO₃

donor culture (intra-erythrocytic parasite culture) maintained below 5% parasitemia by routine subculturing

60% Percoll:

9 volumes of Percoll stock

1 volume of 10× RPMI

5 volumes of 1× RPMI

exflagellation-inducing medium:

10 mM Tris (pH 7.6)

170 mM NaCl

10 mM glucose

10% heat-inactivated normal human serum

25 mM NaHCO₃

50 to 100 μM xanthurenic acid (optional)

Nycodenz (as step gradient of 6%, 11%, and 16%)

Procedure

- Spin down freshly drawn O⁺ human blood (not more than 2 to 4 days old) in 15-mL centrifuge tubes at 1,000 rpm for 10 min. Discard the supernatant including the WBC containing buffy coat. Wash the RBC pellet 3 to 4 times using incomplete medium and resuspend it with an equal volume of incomplete medium to give rise to a final suspension of 50% RBC.
- Start cultures for gametocytes at 6% hematocrit and 0.3% parasitemia. Example: In a 75-cm² flask, add 1.8 mL of 50% washed RBC, estimated volume of parasites from a donor culture, and complete medium to make up the volume to 15 mL.
- Change medium (12 mL) every day. Make sure that the temperature of the medium is 37 °C and culture temperature does not fall below 37 °C during the medium change. A slide warmer set at 37 °C can be used inside the hood to maintain temperature of cultures during medium change.
- Monitor the parasite growth every other day and when parasitemia reaches 3 to 5%, start changing the medium by double the medium volume (25 mL). On the subsequent days keep changing 25 mL of medium on a daily basis.
- It takes around 14 to 18 days to obtain matured gametocyte cultures.

Comments

Highly synchronous stages (I to V) of *Plasmodium falciparum* gametocytes can be obtained by N-acetyl glucosamine treatment of culture (Parasitology 93(Pt 2):263-274, 1986 and Mol Biol Pharmacol 68(1):151-154, 1994).

Enrichment using Percoll centrifugation

- Prepare the 60% Percoll.
- Spin down the parasite culture and resuspend it to ~25% hematocrit in incomplete medium.
- Carefully layer 1 volume of parasite suspension over 2 to 2.5 volumes of 60% Percoll in a 15-mL (13 × 100 mm) polypropylene or Corex glass tube.
- Centrifuge the suspension at 10,000 rpm for 20 min at room temperature.
- Collect parasites at the interphase, wash them 3 or 4 times in incomplete medium, and check them by Giemsa stain.

Gametogenesis and purification of *P. falciparum* gametes/zygotes

Mature gametocytes are stimulated to undergo gametogenesis for 30 min at room temperature (25 to 26 °C) and gametes/zygotes are purified by centrifugation.

- Resuspend mature *P. falciparum* cultured parasites at 20% hematocrit in the exflagellation-inducing medium at a pH of 8.1 to 8.3 at room temperature for 30 min. Addition of 50 to 100 µM xanthurenic acid can also enhance the parasite yield.
- Layer the parasite suspension over a discontinuous step gradient of Nycodenz (6%, 11%, 16%) and spin it at 16,000 × *g* for 15 min at room temperature.
- Gametes/zygotes sediment at the interface of 6% and 11%.

References

Lobo CA, Konings RN, Kumar N. 1994. Expression of early gametocyte-stage antigens Pfg27 and Pfs16 in synchronized gametocytes and non-gametocyte producing clones of *Plasmodium falciparum*. Mol Biochem Parasitol 68(1):151-154.

Ponnudurai T, Lensen AH, Meis JF, Meuwissen JH. 1986. Synchronization of *Plasmodium falciparum* gametocytes using an automated suspension culture system. *Parasitology* 93(Pt 2):263-274.

II:B. Cultivation of *Plasmodium falciparum* gametocytes for mosquito infectivity studies

by **Martin Looker** and **Andrew W. Taylor-Robinson**

School of Biology, University of Leeds, Leeds LS2 9JT, UK

e-mail: m.locker@leeds.ac.uk, a.w.taylor-robinson@leeds.ac.uk

Gametocytes of *P. falciparum* are established from continuously maintained cultures of asexual blood stage parasites. Gametocytes begin to form in significant numbers in blood culture only following daily dilution with fresh RBC and medium for several days. In order for gametocytes to comprise a substantial proportion of PRBC, it is necessary for asexual PBRC to reach a high density and become stressed in vitro before conversion to gametocyte production. This occurs typically 6 to 8 d after the dilution of a culture. Gametocytes then require at least a further 8 d to reach maturity. Cultures for gametocyte production are therefore maintained for a minimum of 14 d without further dilution with fresh RBC in order to obtain a large proportion of mature and infectious gametocytes. We routinely perform gametocyte feeds to mosquitoes using cultures propagated continuously for the preceding 14 to 17 d.

Materials and reagents

Refer to **Appendix** of this protocol for details of materials and generic methods.

Propagation of asexual blood cultures

- Retrieve an isolate of *P. falciparum* cloned line 3D7A from liquid nitrogen and place it in a 25-cm² tissue culture flask along with 0.25 mL of freshly washed RBC and 6 mL of complete medium. Gas the culture for 30 s and tighten the culture flask cap. Keep it in a dedicated 37 °C dry incubator.
- At the same time every day the culture requires a medium change. Tip the flask(s) at 30 degrees to the horizontal, which results in good separation between RBC and the medium that forms the over-layer. As flasks are settled, warm the appropriate volume of complete medium to 37 °C. Settling of flasks and warming of medium takes 40 to 60 min. Remove the spent medium from each culture, discard and replace it with an equal volume (6 mL) of fresh medium, and gas the flask for 30 s. Perform all operations on a warming plate set to 37 °C in order to minimize heat loss to cultures during the time they are out of the 37 °C incubator.

Growth of blood cultures for gametocyte production

- In order to produce gametocytes on a long term basis, it is necessary to maintain a stock of 'asexual' cultures from which the gametocyte cultures are drawn. Every 5 d, flasks which have reached or exceeded a parasitemia of 1% may be subcultured ("split") by adding fresh RBC in order to reduce the parasitemia to about 0.5%. In this way, from one flask at a high parasitemia a number of 'daughter' flasks may be started at a lower parasitemia. Some of these flasks may be used for further stock asexual cultures and some may be used for gametocyte culture. The new asexual stock cultures are grown for another 5 d before being split again when a minimum parasitemia of 1% is reached. This guarantees a continuous supply of cultures for later gametocyte preparation. Existing gametocyte cultures are not subcultured but continue to have their medium changed daily.
- After 6 to 8 d post-subculture, developmental stage I and II gametocytes will start to appear (following asexual PRBC 'stress' once a threshold parasitemia is reached) and from 14 d post-subculture flasks should contain mature, stage V,

- gametocytes. By 17 d, 1 to 4% of PRBC should be stage V gametocytes and the gametocytemia of all stages of development (I-V) may be up to 12%. Progress can be checked by examining thin blood films taken periodically post-subculture (usually on Days 8, 12, and 15).
- Although it is possible to attain much higher gametocytemias by enriching for gametocytes by a variety of treatments (Percoll or Nycodenz density gradient centrifugation or selective drug treatment are the most commonly used), this is not recommended for mosquito infectivity studies. In the experience of ourselves and others, artificially increasing the gametocytemia is often counterproductive to satisfactory transmission of the parasite to the mosquito vector.
 - Development of male sexual stage parasites is more rapid than that of females, so mature male gametocytes may be seen from 14 d but mature females usually only from 16 d post-subculture. In order to ensure a good balance of male and female gametocytes for the purposes of transmission, it is common practice to mix cultures of different ages at the time of the blood feed (see MOSQUITOES AND PARASITES, section II:C).

References

Graves PM, Carter R, McNeill KM. 1984. Gametocyte production in cloned lines of *Plasmodium falciparum*. Am J Trop Med Hyg 33(6):1045-1050.

Ifediba T, Vanderberg JP. 1981. Complete in vitro maturation of *Plasmodium falciparum* gametocytes. Nature 294(5839):364-366.

Read M, Hyde JE. 1993. Simple in vitro cultivation of the malaria parasite *Plasmodium falciparum* (erythrocytic stages) suitable for large-scale preparations. Methods Mol Biol 21:43-55. [Includes useful background information on equipment, reagents, cryopreservation of parasites, preparation of thin blood films, and treatment of contamination]

Appendix

Safety precautions

- *Plasmodium falciparum* is classified as a Category 3 pathogen and all appropriate health and safety measures must be carried out when maintaining this parasite in human blood in vitro, in keeping with local regulations. Perform all work in a Class II safety cabinet. Exclude all nonessential staff from the laboratory while work is in progress. Always wear a Howie-style laboratory coat and latex gloves.
- Any isolate of *P. falciparum* that is used routinely should not be resistant to standard antimalarial drugs.
- Discard spent medium and all disposable plasticware in a hypochlorite disinfectant (e.g., Chlorox or Presept) to a concentration of 10% free chlorine before disposal. Swab all working areas with 70% ethanol at the conclusion of any work. Provide a wash bottle of 70% ethanol to flood any spillage of infective material.

- Obtain blood and serum from a reliable sources only, such as your local Blood Transfusion Service, where it is prescreened for the presence of HIV and hepatitis viruses.

Complete medium

RPMI 1640 medium is purchased as sterile 1-liter bottles (Gibco, 041-91187A). This medium contains 5.96 g/L HEPES buffer, 3.60 g/L glucose, and 50.0 g/L hypoxanthine. To each 1-liter bottle, add 42 mL of freshly prepared filter-sterilized 5% sodium bicarbonate (Sigma S-5761) (this is referred to as 'incomplete' medium). Following the further addition of 100 mL of heat-inactivated pooled human serum, the liquid is now 'complete' medium. Store the medium at 4 °C and use it within 7 d, during which time the pH should be monitored (optimum pH 7.3).

Retrieval of parasites from liquid nitrogen

- Thaw cryotube(s) in a 37 °C water bath for 2 min.
- Transfer the thawed contents to a sterile microcentrifuge tube and centrifuge it at $10,000 \times g$ for 1 min, then remove the supernatant.
- Resuspend the pellet in 1 mL of PBS + 10% sorbitol; add this slowly dropwise with continuous mixing.
- Repeat steps 2 and 3 twice more (i.e., total of 3 washes).
- Wash and resuspend the pellet in complete medium when the cells are ready for culture.

Washing human RBC

- Use group O, Rhesus group positive whole blood taken into adenine-CPD (citrate phosphate dextrose). Blood used should be less than 7 d old.
- Centrifuge the blood at $800 \times g$ for 5 min.
- Remove the white blood cells by aspiration of the buffy coat.
- Add an equal volume of incomplete medium and centrifuge it at $800 \times g$ for 5 min.
- Discard the incomplete medium.
- Repeat steps 4 and 5 twice more (i.e., total of 3 washes).
- After washing, the packed RBC may be used directly (i.e., at a 100% hematocrit) and any remainder discarded. Alternatively, the packed RBC may be resuspended in an equal volume of complete medium (i.e., at a 50% hematocrit), which should be stored at 4 °C and used within 7 d.

Gas mixture

1% oxygen, 3% carbon dioxide, and 96% nitrogen (BOC Specialty Gases, or your local specialist gas supplier). Pass the gas mixture from the cylinder via a gas-reducing valve into the safety cabinet by a silicon tube. Filter the gas through an in-line Whatman gamma 12 sterilizing unit with a 0.3- μ m filter tube. Connect the filter to a sterile Millex GS 0.22- μ m disc filter, the end of which is attached to a 19G $\times$ 1.4-inch blunt-ended needle fitted to a 1-mL syringe. When gassing a culture flask, the flow rate should be moderate but sufficiently strong to ruffle the surface of the liquid.

Pooled serum

Pool human group O, Rhesus group positive serum from a minimum of 10 donors to minimize variations of quality between individuals. Packs of serum are routinely heat-inactivated at the source, but if not they should be held at 56 °C for 1 h. Test the pools

before full-scale use to ensure that they are sterile and support parasite growth, in particular that of gametocytes. Aliquots may be stored at -20°C for up to 6 months.

Parasites

In order to maximize the likelihood of satisfactory gametocyte production, it is best to use a line or clone of *P. falciparum* that is known for its production of gametocytes. Good producers include NF54, 3D7A, and HB3A, obtainable from the MR4 repository. Restart cultures from frozen stocks every 2 months or less since gametocytogenesis in a continuously cultured line will start to wane after this period.

Parasite staining

Use improved R66 Giemsa (BDH 350864X) diluted 1:10 with Sorensen's buffer (HD Supplies; HDS 20, pH 7.2). Fix thin blood films in 100% methanol and stain for 20 min in the 10% Giemsa solution. Wash them with tap water for 20 s and allow to air dry before viewing by light microscopy using oil immersion at 1000 $\times$ magnification.

Thin blood film

Small volume of RBC removed from culture flask and smeared across a glass slide to give a monolayer of RBC.

Sterile plasticware

- 25-cm² cell culture flasks (Iwaki 3100-025 or similar)
- 30-mL Universal containers (Sterilin 128A or similar).
- plastic pipettes, singly wrapped, disposable, graduated (2-, 5- and 10-mL; Sterilin or similar)

II:C. Production of *Plasmodium falciparum* oocysts and sporozoites**by Martin Looker and Andrew W. Taylor-Robinson***School of Biology, University of Leeds, Leeds LS2 9JT, UK**e-mail: m.locker@leeds.ac.uk, a.w.taylor-robinson@leeds.ac.uk*

The production of different sexual stages of *P. falciparum* and their isolation from infected mosquitoes facilitates a variety of cellular, molecular, immunochemical, and transmission-blocking studies that examine the parasite-vector relationship. The procedures of mosquito rearing, parasite cultivation, and infection and dissection of mosquitoes are highly specialized skills requiring considerable experience to perform with competence. Additionally, the timing of the two life cycles, that of the parasite and that of the mosquito, requires precise planning so that the mosquitoes are the correct age to infect during the brief period in which the parasites are viable for infection.

Mosquitoes capable of transmitting *P. falciparum* should be maintained within a limited access, dedicated, high security humidified insectary. It is recommended that all rooms be painted in a light color and all cages be constructed of white materials in order to make highly visible any mosquito that may temporarily escape. A nonresidual insecticide spray should also be available in the event of an emergency. Infected mosquitoes to be dissected are first stunned or anesthetized by chloroform and then rendered incapable of movement (dewinged and delegged). Any potentially infective mosquitoes that are not to be dissected are frozen by placing the complete container at -20°C prior to autoclaving and disposal.

Equipment required for blood feed of mosquitoes

(A) *Pooter*—A pooter is a device used to transfer mosquitoes by aspiration. We use a polycarbonate plastic-tubed pooter, but any toughened material is suitable provided that it is transparent. Ours are custom-made (contact details upon request) but an efficient pooter can equally well be modified from a 10-mL plastic pipette. This is prepared for use as follows:

- Insert a stainless steel (or nylon) fine-meshed disc of the internal dimensions of the pooter tube 2 cm from one end of a 20- to 25-cm tube. A ring may be etched in the internal surface of the tube in which the disc can sit securely.
- Attach a length of clear, transparent silicon rubber tubing (approximately 30 cm) over the meshed end of the polycarbonate tube.
- Use the pooter by placing the free end of the silicon rubber tubing in your mouth and aspirating by a 'suck-blow' technique. Transfer the mosquitoes by sucking them into the open end of the polycarbonate tube and then blowing them out into the container.

(B) *Mosquito container*—The ideal container should minimize the risk of mosquito escape or injury or of the handler being bitten. The container used successfully by ourselves and several other research groups is customized from a 500-mL cylindrical ice-cream container made of waxed cardboard (of the type used by Ben & Jerry's and Häagen-Dazs). This is prepared for use as follows:

- Cut out a circular piece of filter paper (Whatman no. 1, or similar) to the internal dimensions of the container and tape it to the floor of the container to soak up any spillage that may occur (from nutrient feed, diuresis drops, etc.) when in use.
- Cut a 3-cm square in the side and cover the hole with 2 layers of dental latex (HCM rubber dental dam or similar, available from your local dental supplies distributor) into which two 1-cm cuts are made, one of which is then placed

perpendicular to the other. Hold both squares of latex firmly in place with electrician's insulating tape. This results in a cross-shaped entry port through which, with the aid of a pooter, mosquitoes can be safely introduced or removed.

- To complete the container, secure a double layer of 'bridal veil' netting over the mouth of the container with elastic bands and insulating tape.
- Other than during the actual blood feed, keep the containers inside a gauze-covered cage that acts as a secondary security barrier.

(C) *Blood feeder*—A glass membrane feeder is used to deliver the infectious blood meal to the mosquitoes. Blood feeders consist of a water-jacketed chamber into which the infectious blood feed can be introduced. Ours are custom-made by a professional glass blower (contact details upon request). This is prepared for feeding use as follows:

- Cover the blood feed chamber with a Baudrouch membrane derived from bovine intestine (Joseph Long Inc.). Wet the membrane with tap water to ensure that, when dry, it is taut. This will encourage the mosquitoes to feed. If washed and dried thoroughly after each use, a membrane can be used 5 or 6 times before it loses its integrity. In fact, in our experience, previously used membranes are preferred to new membranes because their use results in a greater proportion of mosquitoes taking a blood meal.
- Hold the membrane in place with an elastic band.
- Pass tap water at 38 °C through the water jacket via a recirculating pump (water inlet via the lower tube, outlet via the higher tube).

Rearing of *Anopheles stephensi* mosquitoes

- Keep each stock colony of *An. stephensi* in a gauze-covered, plastic-covered wire-framed cage. We use cages of frame dimensions 30 cm³, which are covered with mosquito netting or 'bridal veil' netting to incorporate a 15-cm front sleeve.
- Keep cages at 26.0 ± 0.5 °C and 80.0 ± 2.0% relative humidity. Maintain all colonies on a reverse light cycle with 12 h artificial lighting from 2000 to 0800 h. This enables all manipulations to be performed in keeping with the nocturnal activity of the mosquito during normal working hours.
- Feed adult female mosquitoes twice a week on uninfected rodent blood. We routinely feed directly by bite of naive outbred mice, but rats or guinea pigs are also suitable. Anesthetize the mouse by intraperitoneal injection of 0.1 mL per 10 g body weight of 10% Hypnorm (Janssen Pharmaceutica) and 20% Valium (Roche) in sterile distilled water. Place the mouse abdomen-down, with the four limbs stretched out, on top of the cage in which the mosquitoes to be fed are kept, and allow feeding for 20 min. The abdomen may be shaved of fur to facilitate access for feeding. We use mice under terminal anesthesia in accordance with project and personal licences issued by the UK Home Office and following institutional guidelines. Your local regulatory authority may permit rodents to be reused for the purposes of mosquito feeding, in which case they can be allowed to recover from the anesthetic.
- Eggs are laid by fed female mosquitoes 2 to 3 d after a blood feed. Place a small bowl lined with filter paper (Whatman no. 1, or similar) and filled with tap water on the floor of the stock cage overnight. The following morning, remove the bowl to another cage, and cover it with a square of transparent Perspex until the eggs begin to hatch (within 2 d).

- Rear the larvae in clean plastic washing-up bowls (preferably colored white) containing 3 inches of water. Carefully transfer the contents of the hatching bowl into a larval rearing bowl (approximately 300 larvae per bowl) and discard the filter paper. The water must not contain large amounts of chlorine so it may be necessary to allow your local tap water to stand for a minimum of 24 h before use to allow any chlorine to dissipate ('chlorine-free' water). We recommend the continuous maintenance of a reservoir of chlorine-free water ready for use. Alternatively, water can be boiled before use or bottled water can be used. Whatever the source and treatment of the water, it must be at room temperature before eggs are added.
- Anopheline mosquito larvae are primarily surface feeders and must be provided with a form of food that floats on water. We use koi carp pellets (Wardley Corporation premium koi staple, or a suitable alternative supplied by your local aquarist). Baby weaning formulation milk powder ('Farex' or similar) may also be used but should be finely sieved before sprinkling on the water.
- Feed the larvae morning and evening with fresh pellets (a 'little but often' regime). Remove and discard any surplus 'old' food. In this way, the larvae receive sufficient food, but not so much that the bowl becomes contaminated with uneaten food.
- After 2 to 3 d, reduce the mosquito numbers. By keeping the larvae numbers between 200 and 250 per bowl, large well-nourished mosquitoes are produced.
- Larvae should pupate 6 to 8 d post emergence from eggs if kept at 26 °C and 80% relative humidity. Pupation can be delayed by 2 or 3 d by reducing the ambient temperature by 1 or 2 °C. Kill surplus larvae by immersion in hot water. Do not discard them live down the sink.
- Collect pupae and place them in a bowl of chlorine-free water. Cut an inch off of the point of a filter-paper cone and place the cone over the bowl. This system allows emergence of adult mosquitoes (2 to 3 d postpupation), but also deters emergent mosquitoes from flying back into the bowl and drowning and keeps fed females from laying eggs in the pupae bowl. Place the pupae bowl in the stock cage.
- Provide a solution of 5% glucose (Sigma) and 0.05% *p*-aminobenzoic acid (PABA; Sigma) in chlorine-free water for all adult mosquitoes. Filter-sterilize this nutrient solution through a 0.22- μ m filter (Sterilin or similar) before soaking a cotton wool pad and placing it in the cage. The pad should be moist but not dripping as this leads rapidly to fungal contamination, and should be changed daily.

On the day before the feed

- Prepare the mosquito container(s) and place into each, with the aid of a pooter, 4 to 6-d-old virgin female mosquitoes (i.e., they should be 5 to 7 d postemergence from pupae on the day of the feed). Add a suitable number of mosquitoes for the volume of the container to be used; for a 500-mL cylindrical container, we add between 100 and 250 mosquitoes. Up to 20 mosquitoes can be accommodated comfortably within a pooter at any one time to enable counting during the transfer procedure. It is important to handle the mosquitoes carefully so as not to damage them.
- Test mosquitoes for their readiness for a blood feed by evaluating their attempt to feed on a warmed, water-filled, tissue culture flask placed on top of the gauze-

- covered stock cage in which they are housed. If the pooter is used for transfer to the feed container while the culture flask is in place, those mosquitoes that are attracted by the warm object can be positively selected. This procedure acts not only to select older individuals more eager for blood among a population of slightly mixed ages (due to variation in the timing of emergence from pupation) but also to segregate female from male mosquitoes. Only females are attracted by the warmed flask, as its temperature mimics that of a human (from which they require blood in order to lay eggs); male mosquitoes do not blood feed and are ambivalent to the presence of the flask.
- Feed mosquitoes on a cotton wool pad soaked in 5% glucose/0.05% PABA which is placed on top of the mosquito container. Remove the cotton wool pad 12 h before the blood feed. Starving the mosquitoes for 12 h prompts the vast majority to feed to engorgement on blood.

On the day of the feed

- Wash fresh group O, Rhesus group positive whole blood and warm it to 37 °C.
- Warm group O, Rhesus group positive serum to 37 °C.

For details of preparation of human RBC and pooled serum, refer to the Appendix of MOSQUITOES AND PARASITES, section II:B.

- Place a Baudrouch membrane on the blood feeder, wet it, and allow it to dry. Once the membrane is dry (approximately 20 min), switch on the water pump and allow the water jacket to warm up to 38 °C (approximately 60 min).
- Take *P. falciparum* to be used for the feed from 14- to 17-d-old cultures which contain mature, stage V gametocytes. As male gametocytes mature more rapidly than do females, in practice it is best to use either: a) exclusively cultures that are 17-d post-subculture, which should contain a sufficient number of gametocytes of both sexes; or b) a preparation of blood of different ages (such as a 1:1 mix of 14- and 16-d-old cultures), which will show a sex ratio bias in favor of male or female parasites, respectively. For method of cultivation, refer to “Growth of blood cultures for gametocyte production” of MOSQUITOES AND PARASITES, section II:B.

If at all possible, perform the following procedures entirely at 37 °C and as rapidly as safe handling and good microbiological practice permits in order to minimize the possibility of gametocytes committing to activation/exflagellation before the blood meal is taken up by the mosquito.

- Transfer infective cultures to centrifuge tube(s) prewarmed to 37 °C.
- Centrifuge the cultures at $800 \times g$ for 2 min in a centrifuge prewarmed to 37 °C.
- Remove and discard the supernatant.
- Measure the volume of the remaining pellet, containing packed PRBC, and add an equal volume of serum.
- Make the volume of infective blood/serum from step 8 up to that of the blood feeder to be used (1 to 2 mL) by diluting the suspension up to 3 $\times$ volume with a mixture of 1:1 washed human RBC/serum. Mix gently but thoroughly. We routinely dilute blood in this manner because: a) better infection rates are achieved using blood that contains a high but submaximal gametocytemia; b) addition of fresh blood/serum to the 17-d-old cultured PRBC promotes mosquito

- feeding, in terms of both extending the feeding time of individual mosquitoes (qualitative) and increasing the number that feed (quantitative).
- Rapidly transfer the diluted infective blood from step 9 into the blood feeder using a prewarmed syringe with a blunt-ended needle, ensuring that there are no air bubbles present.
 - Remove a mosquito container from its outer cage. Place the container underneath the blood feeder, ensuring that the blood feeder and the netting are in intimate contact.
 - Turn off any artificial lighting and allow the mosquitoes to feed undisturbed for 12 to 15 min. We recommend that the high security insectary in which the mosquitoes are held be without windows; this enables feeding to take place in the dark, mimicking feeding behavior in the wild.
 - Remove the mosquito container from underneath the blood feeder, thus terminating feeding, and return it to a gauze-covered cage. Most mosquitoes that have been starved of nutrients for the previous 12 h will feed to engorgement within a few minutes. These will have a very distended, red abdomen and can be easily distinguished from mosquitoes that have not fed. If the mosquitoes have fed well, the floor of the container will be covered with red splashes of fluid passed by diuresis. Depending on the ease with which the pooter can be manipulated within the container, it may be possible at this stage to remove individual unfed mosquitoes.
 - During the time the blood feed is taking place, examine any infective blood/serum mix that was not added to the membrane feeder for gametocyte activation and/or exflagellation. Exflagellation is the explosive production of male gametes of the malaria parasite, which happens in the mosquito midgut within a few minutes of a blood meal. The cytoplasm of the male gametocyte becomes agitated, followed rapidly by the sudden and vigorous protrusion and detachment of up to 8 flagellated gametes (spermatozoa) from the surface of the parasite cell body. This phenomenon also occurs spontaneously in vitro and thus may be observed in cultures of fresh infected blood under the light microscope (1000× magnification under oil immersion). It is controlled in vitro solely by the change from 37 °C to the ambient laboratory temperature, the pH rise this brings being mediated by a fall in CO₂ tension as the blood equilibrates with the atmosphere. Viewing of gametogenesis (typically between 10 and 25 min after removal of a blood culture from the 37 °C incubator) is a strong indicator that oocysts and sporozoites will be produced following a blood feed. However, in our experience, observable exflagellation is not a prerequisite for successful mosquito infectivity.

On days after the feed

- Subsequent to a blood feed, feed mosquitoes daily on a 5% glucose/0.05% PABA diet, as previously described. Delaying the start of feeding by 24 h post blood feed enriches for blood-fed mosquitoes as the vast majority of 'die off' mosquitoes under these conditions are those that did not blood feed and therefore were starved of nutrients for at least 36 h.
- Mosquitoes may be dissected for the presence of oocysts at 10 to 12 d postblood feed. Dissection for sporozoites may take place from 14 d postblood feed. The number of live mosquitoes in each container at the time of dissection must be verified and recorded by 2 people independently (usually by observing carefully the pooter process). The total number of mosquitoes either dissected or

undissected at the end of the experiment should tally with the records made. The protocol for dissections is described below.

Dissection of blood-fed mosquitoes

Dissection of mosquito midgut to determine the presence of oocysts

- For the detection of *P. falciparum* oocysts, dissect mosquitoes 10 to 12 d postinfective blood feed.
- Remove mosquitoes from the container via a pooter, the end of which is immediately plugged with cotton wool and sealed with masking tape to prevent escape. Mosquitoes may be collected one at a time, but up to 5 mosquitoes can be held in the cavity of the pooter without compromising handler safety.
- Strike the pooter against the palm of the hand firmly several times to knock the mosquitoes out for a sufficient period of time to allow their safe removal onto a dissection board. In order to stop each mosquito from flying, and also as an aid to dissection of the body, remove its legs and one wing with a scalpel blade.
- Place dewinged and delegged mosquitoes in a watch glass containing dissection medium (PBS + 0.0001% FCS). Using a pair of fine forceps, pick up each mosquito by its one remaining wing and dip it into 70% ethanol to ensure both sterility and killing of the mosquito.
- Remove individual mosquitoes, mount them on glass microscope slides onto which a drop of dissection medium had been previously placed, and remove the remaining wing.
- Dissect the mosquito under a binocular light microscope using a 40× objective (total magnification 400×).
- Separate the abdomen from the rest of the mosquito. Remove the gut by holding the anterior of the abdomen with one dissecting needle while at the same time making a small cut with a second needle in the tegument on each side of the seventh abdominal segment. Using the second needle, gently pull on the apex of the abdomen until the gut and Malpighian tubules are exposed.
- Sever the alimentary canal sufficiently far forward to bring away (and discard) all of the foregut except for the section immediately proximal to the midgut.
- Anchor the remainder of the gut by placing the point of one needle on the posterior section of foregut. Using the other needle, cut through the alimentary canal at the junction of the midgut and the hindgut, simultaneously severing the Malpighian tubules. Discard the hindgut and Malpighian tubules, leaving only the midgut on the slide.
- When an especially rapid dissection is required (with least chance of disruption to the oocysts), once the gut has emerged completely from the abdomen, cut off and remove the hindgut only, leaving the midgut exposed but with the foregut and esophagus still attached.
- Using a needle, pick up the dissected midgut and transfer it to a fresh slide onto which has been placed a drop of dissecting medium. Lower a coverslip gently onto the moistened midgut, which then can be viewed for the presence of oocysts. The volume of medium bathing the preparation is important as too little will cause the midgut to rupture (so releasing oocysts and thereby preventing their examination *in situ*) and too much will prevent adequate flattening of the

- midgut for optimal viewing. Draw off excess medium by holding a filter paper to the edge of the slide.
- View guts by under 400× magnification with reduced light when oocysts, if present, should be easily identified as circular refractive bodies on the gut wall of the dissected mosquito.
 - In our experience, the number of oocysts recovered per infected mosquito varies between 1 and >200, average 30. Mosquitoes that are not infected, as determined by a lack of oocysts, are usually those that did not blood feed and will not contain eggs. Occasionally, an infected mosquito (with oocysts) that has no eggs may also be seen.

Dissection of mosquito salivary glands to determine the presence of sporozoites

- Consider freshly dissected salivary glands containing *P. falciparum* sporozoites as potentially infectious and handle them with appropriate caution. Always wear a Howie-style laboratory coat and latex gloves.
- Sporozoites may first be observed in the salivary glands 14 to 17 d postinfective blood feed. The junction of the mosquito head and thorax contains a pair of glands, each of which is tri-lobed (or very occasionally four-lobed).
- Repeat steps 1 through 6 as described above for oocyst dissection.
- Place a dissecting needle gently on the thorax of the mosquito, just below the region where the salivary glands lie.
- Place a second needle on the 'neck' of the mosquito (apex of head and thorax) without cutting it, then with a gentle pulling action detach the head.
- The salivary glands should now be exposed and can be detached from the head. If the salivary glands are not apparent, then press with a needle again on the thorax, when the salivary glands should emerge from the thorax and can be detached.
- Transfer the dissected glands and a small volume of dissection medium into a 0.5-mL microcentrifuge tube using a 100- to 200-μL Gilson pipette. Keep the microcentrifuge tube on ice.

Liberation of sporozoites from salivary glands

- Cover the microcentrifuge tube containing the salivary glands with Parafilm to reduce the chance of leakage. Release the sporozoites by gently rupturing the glands using a laboratory benchtop whirlimixer set to maximum for 3 min or by vigorous pipetting using a 200-μL Gilson pipette.
- Once liberated from the salivary glands, the sporozoite yield can be estimated by counting using an hemocytometer. Place 10 μL of the sporozoite suspension into the hemocytometer chamber and then using 200 to 400× magnification phase-contrast microscopy to visualize the sporozoites, count the number present in a 4 × 4 grid: this number relates to the number of sporozoites per mm² × 10⁴.
- If a qualitative examination for sporozoites is all that is required, transfer salivary glands directly to a glass microscope slide. Lower a coverslip carefully onto the moistened glands and tap it gently to disrupt the glands and release any sporozoites present. These should be easily identified as comma-shaped, motile bodies.
- In our experience, the number of sporozoites recovered per infected mosquito varies between 4,000 and 30,000, average 15,000.

References

Taylor-Robinson AW, Looker M. 1998. Modulatory effect of nitric oxide on *Plasmodium falciparum* male gametocyte exflagellation. *In*: Moncada S, Toda N, Maeda H, Higgs EA, eds. The biology of nitric oxide, part 6. London: Portland Press; p. 248.

Taylor-Robinson AW, Looker M. 2000. Nitric oxide-mediated modulation of *Plasmodium falciparum* infectivity for the mosquito vector of malaria. *In*: Moncada S, Gustafsson LE, Wiklund NP, Higgs EA, eds. The biology of nitric oxide, part 7. London: Portland Press; p. 184.

III. Transmission blocking and sporozoite invasion assay

III:A. Transmission blocking assay (TBA) – membrane feeding

by **Mrinal Kanti Bhattacharyya** and **Nirbhay Kumar**

Kumar Lab, Johns Hopkins Malaria Research Institute, Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, USA
e-mail: nkumar@jhsph.edu

Equipment

membrane feeder (glass), Lillie Glassblower, USA (Tel: + 1-404-436-8959)
silicon tubing to connect the feeders
aspirator tube
pint containers (Naptune Brand Round containers, Kahn Paper Co.)
dental dam, hygienic
nylon net, bridal net (any fabric store).
Parafilm
water pump, constant temperature circulator (Polystat, Cole-Parmer, or any other equivalent)
light microscope (e.g., Olympus)
dissecting microscope (e.g., Olympus)
dissecting forceps
dissecting glass slide
glass slides and coverslips
Eppendorf tubes
sterile pipettes
insectaries approved for handling *Plasmodium falciparum*-infected mosquitoes

Materials and reagents

parasite culture:
Maintain *Plasmodium falciparum* gametocyte culture for 14 to 18 days making blood smears on alternate days. From Day 12 onward, make blood smears on a daily basis and look for mature C-shaped gametocytes as well as in vitro exflagellation of male gametocytes.
mosquitoes:
Maintain and breed *Anopheles stephensi* according to the standard procedure (see MOSQUITOES AND PARASITES, section II:C). Use 3- to 5-d-old mosquitoes for the experiment.
human RBC
heat-inactivated normal human serum (NHS)
heat-inactivated antiserum
incomplete medium used for *P. falciparum* cultures
mercurochrome solution

Methods

Important notes before starting

- *Plasmodium falciparum* cultures of at least 0.5% mature gametocytes and at least 1 to 2 exflagellation centers per field should be used for mosquito feeding.
- Before preparing blood meal, have mosquitoes and feeders ready.
- Take care to maintain the parasite and the blood always at 37 °C.

Exflagellation assay

- Using a sterile pipette, take out 1 mL of parasite culture and transfer it to an Eppendorf tube.
- Spin down the tube for 15 s at high speed and discard the supernatant.
- Add an equal volume of normal human serum to resuspend the pellet and allow it to stand for 10 min at room temperature.
- Place 10 μ L of this suspension on a glass slide and cover it with a coverslip.
- Determine the average number of exflagellation centers per field using a light microscope at 400 $\times$ magnification. A polarizer filter may help in locating the parasitized RBC as in this configuration the malaria pigments look shiny. Scan at least 10 to 20 fields to get a proper estimate of exflagellation centers.

Making dilution of antibodies in 50% RBC

Premix the antibodies to be tested for transmission blocking activities with 50% red blood cells in heat-inactivated normal human serum at various dilutions (1:5 to 1:20).

Keep these diluted antibodies at 37 °C until the final feeding.

- Place 100 μ L of 50% RBC (in incomplete medium) in a microfuge tube, harvest the cells at 3,000 rpm for 1 minute, and wash them with heat-inactivated NHS.
- Resuspend the cells at 50% hematocrit in NHS and antiserum to obtain desired serum dilution.
- Incubate the tubes at 37 °C.

Preparation of mosquito pint

- Make a hole (2-cm diameter) in the paper can. Cover the hole with two squares (2 in $\times$ 2 in) of dental rubber with perpendicular cuts on top of each other using adhesive tapes.
- Cover the top of the paper can with nylon net of appropriate size and securely tape the net to the paper can.

Collecting female mosquitoes in pint

Distribute 30 to 40 female mosquitoes per pint. The easiest way to collect female mosquitoes from a mixed pool of male and female is to place a warm water bottle (39 to 40 °C) against the wall of the mosquito container. The female mosquitoes are attracted to the warmer side of the cage, whereas the male mosquitoes will be all over. Starve the mosquitoes for 5 to 6 h.

Preparation of feeders

- Connect the glass feeders to each other and to the circulation water pump with silicon tubing to generate a continuous flow of 39 °C hot water through the water jackets of the membrane feeders.
- Stretch Parafilm membrane (approximately 2 in $\times$ 2 in) in both directions and place on the glass feeder. Maintain the temperature of the membrane feeder by a glass water jacket (39 °C) and a circulating water bath.
- Place the pint with female mosquitoes under the warmed membrane feeder.

Membrane feeding

This step is the most crucial and must be performed as quickly as possible. During the whole procedure, take care not to allow the temperature of the *Plasmodium falciparum*

gametocyte culture to drop below 37 °C. A drop in temperature promotes exflagellation. The success of the experiment depends on the maximization of exflagellation as well as gametogenesis within the mosquito, not outside. Allow the mosquitoes to engorge for 15 min.

Experimental steps

- Place 10 mL of matured *Plasmodium falciparum* gametocyte culture in a 50-mL conical tube.
- Harvest at 1,000 rpm for 2 min and then allow the rotor to stop naturally (without the break).
- Remove the supernatant and resuspend the pellet at 50% hematocrit with prewarmed NHS.
- Keep the tube warm at 37 °C.
- Lower the membrane feeder over the nylon net covering the pint. Make sure that the mosquitoes are attracted to the membrane.
- Add 50 µL of gametocyte suspension kept warm at 37 °C to the diluted antibody and immediately transfer the mixture to the feeders.
- After 15 min, separate the unfed mosquitoes from the fed ones. It is very easy to separate the fed mosquitoes from unfed mosquitoes as the fed mosquitoes have swollen red abdomens.
- Feed sugar and water to the mosquitoes and maintain them for 7 to 9 more days in a room maintained at 26 °C and 60 to 80% relative humidity.

Scoring and statistical analysis

Perform any TBA experiment with at least 2 or 3 different concentrations of the test serum. The controls may include preimmune sera, irrelevant antisera (raised against asexual stage specific antigens), no antiserum added, and a serum known to possess transmission blocking activity.

- After 7 to 9 days, dissect the midgut of each and every individual mosquito from different groups under the dissection microscope and look for the presence of oocysts. Mosquitoes can be knocked down by dropping them in 70% ethanol and then transferred to a container with 1× PBS. Transfer the mosquitoes to glass slides and carefully pull the posterior part of mosquito with dissecting forceps, holding the anterior part of mosquito at the same time with another forceps. Treat the mosquito midguts thus separated with drops of 0.1% Mercurochrome solution and allow them to stain for a few minutes under coverslips.
- The oocysts will stain red on a light pink background of the midgut. Count the number of oocysts per midgut.
- Calculate the infection rates (number of mosquitoes infected per total number dissected) and number of oocysts (geometric means) in mosquitoes.
- Compare the infection rates and the number of oocysts in mosquitoes fed test serum to those obtained from feeds using appropriate control sera.
- Perform the Mann–Whitney test to assess the statistical significance.

Comments:

Apart from assessing the potency of different transmission blocking antibodies, the same experimental procedure can be used to address other biologically important questions. For example, effects of some chemotherapeutic drugs as transmission blocking agents

can easily be scored with the help of this assay. Also the involvement of different agents on parasite infectivity can be quantitated. A similar experimental design is also used to perform genetic crosses using different parasite strains.

Reference

Bhattacharyya MK, Kumar N. 2001. Effect of xanthurenic acid on infectivity of *Plasmodium falciparum* to *Anopheles stephani*. Int J Parasitol 31(10):1129-1133.

III:B. Sporozoite invasion assayby **Christopher J. McCormick¹**, **Michael R. Hollingdale²**, and **Andrew W. Taylor-Robinson¹**¹*School of Biology, University of Leeds, Leeds LS2 9JT, UK**e-mail: c.j.mccormick@leeds.ac.uk, a.w.taylor-robinson@leeds.ac.uk*²*London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK**e-mail: michael.hollingdale@lstmh.ac.uk*

The techniques used for assessing *Plasmodium berghei* and *P. falciparum* sporozoite invasion are identical, although the latter parasite obviously poses a substantial risk to any individual exposed to it, so due care should be taken in handling infectious mosquitoes and viable sporozoites. Sporozoites are layered over hepatoma (HepG2-A16) cells or primary human hepatocytes and recognized by monoclonal antibody or serum raised to circumsporozoite (CS) protein, which bind the cell surface of sporozoites and block sporozoite invasion into hepatoma cells in vitro.

The reagent that is recommended for sporozoite detection is an IgM monoclonal antibody (MAb 36) which reacts with the CS proteins of both *P. falciparum* and *P. berghei*, and which was isolated from *P. falciparum* sporozoite-immunized mice (Sina et al. 1992; available on request from MR Hollingdale). In assays of biological activity, MAb 36 induces the CS precipitation reaction with live sporozoites and blocks the invasion of hepatoma cells by sporozoites in vitro at concentrations much lower than those observed for other reported CS protein-specific MAb. However, suitable alternatives are available in the MR4 repository: rabbit antiserum against *P. falciparum* CS protein (MRA-24) and rat antiserum against recombinant *P. falciparum* CS protein region I to C-terminus (MRA-21).

Equipment

CO₂-incubator
200-μL Gilson pipette
hemocytometer
phase-contrast microscope

Materials and reagents

immortalized human hepatocyte cell line HepG2 (available from MR4 as either a frozen ampoule or a growing culture)
entacin–collagen–laminin (ECL)
serum-free RPMI 1640 medium
mosquitoes (see MOSQUITOES AND PARASITES, section II:C)
PBS
PBS + 0.0001% FCS
PBS + 1% BSA
MEM; minimal essential medium (Sigma)
10% heat-inactivated FCS (referred to as 'binding medium')
100% methanol
MAb 36
goat anti-mouse peroxidase conjugate (DAKO)
distilled water
DAB tablet (Sigma 'Fast' DAB kit)
H₂O₂ (Sigma 'Fast' DAB kit)
DPX mountant (HD Supplies)

8-well Nunc slides

Producing a confluent HepG2 monolayer to study sporozoite invasion

The growth of HepG2 cells on 8-well Nunc coverslips is achieved using standard culture techniques, splitting confluent cultures between 1:3 and 1:6. However, the surface of slide wells should be precoated with an extracellular matrix (entacin–collagen–laminin; ECL) to promote cell attachment.

- Dilute a 40- μ L frozen aliquot of ECL (stock concentration at 1 mg/mL) in 4 mL of serum-free RPMI 1640 medium. Add 200 μ L of this solution to each well of the 8-well slides.
- Leave at 37 °C for a minimum of 2 h.
- Aspirate off the ECL solution and add a 250- μ L suspension of HepG2 cells to each well.
- Place slides in an incubator (37 °C, CO₂) for 2 to 3 d to allow the cells to attach and grow before using them in an invasion assay.

Invasion assay

Consider freshly dissected salivary glands containing *P. falciparum* sporozoites as potentially infectious and handle them with appropriate caution. Always wear a Howie-style laboratory coat and latex gloves.

- Dissect the mosquitoes for sporozoites between 14 and 17 d after an infectious feed, as described in detail previously (see MOSQUITOES AND PARASITES, section II:C).
- Dissect out both salivary glands from the apex of head and thorax of each mosquito and remove any unwanted body tissue. Collect salivary glands into ice-cold PBS + 0.0001% FCS. In our experience, the number of sporozoites recovered per infected mosquito varies between 4,000 and 30,000, average 15,000.
- Using a 200- μ L Gilson pipette, vigorously pipette the suspension containing mosquito salivary glands up and down to disrupt the glands in order to release free sporozoites.
- Determine the density of sporozoites in this suspension using a hemocytometer. Place 10 μ L of the sporozoite suspension into the hemocytometer chamber, use phase-contrast microscopy to visualize the sporozoites, and count the number present in a 4 $\times$ 4 grid. This number relates to the number of sporozoites per mm² $\times$ 10⁴.
- Dilute and divide the sporozoite suspension so that there are between 10,000 and 20,000 sporozoites per 300 μ L of minimal essential medium (MEM) + 10% heat-inactivated FCS (referred to as 'binding medium'). Ensure that this density is the same for all groups that are to be compared.
- Remove 8-well slides containing HepG2 cells from the 37 °C CO₂ incubator, wash each well once with 300 μ L of binding medium, and add 300 μ L of sporozoite suspension. Place slides in a loose-lid container and return them to the 37 °C CO₂ incubator for 3 to 4 h.
- Gently aspirate off the sporozoite suspension, add 300 μ L of warmed MEM (without FCS), and leave the slides for 5 min.
- Repeat the wash.
- Aspirate off the MEM and flood the wells with 100% methanol. After 5 min, remove the methanol, add PBS, and leave them at 4 °C overnight.

Visualization of sporozoites

Visualize the sporozoites using immunohistochemical techniques.

- Discard the PBS solution and remove the plastic well surrounds and the rubber gaskets from the 8-well slides.
- Block each monolayer with a 100- μ L solution of PBS + 1% BSA for 10 min.
- Aspirate off the solution and add 100 μ L of PBS + 1% BSA + 5 μ g/mL of MAb 36 (shows immunological cross-reactivity between *P. falciparum* and *P. berghei* CS proteins).
- Incubate the slides in a humidified chamber at room temperature for 30 min.
- Aspirate off the primary antibody solution and wash the cell monolayers by placing the slides in a Coplin jar containing PBS for 2 min. Repeat this step twice more.
- Add 100 μ L of PBS + 1% BSA containing a 1:200 dilution of goat anti-mouse peroxidase conjugate. Incubate the slides in a humidified chamber at room temperature for 30 min.
- Repeat the PBS washes.
- In 1 mL of distilled water, dissolve one DAB tablet and one H₂O₂ tablet from the Sigma 'Fast' DAB kit. Add 100 μ L of this solution to each cell monolayer. Allow the reaction to proceed for 2 min and then terminate it by washing off the DAB solution with tap water. Note: **DAB is very toxic**. Always wear gloves when handling it and use copious amounts of water to wash solutions containing DAB down a suitable laboratory sink.
- Allow slides to dry and then mount them using DPX mountant and glass coverslips. Visualize sporozoites under either 200 $\times$ or 400 $\times$ magnification using phase contrast microscopy.

References

McCormick CJ, Tuckwell DS, Crisanti A, Humphries MJ, Hollingdale MR. 1999.

Identification of heparin as a ligand for the A-domain of *Plasmodium falciparum* thrombospondin-related adhesion protein. Mol Biochem Parasitol 100(1):111-124.

Sina BJ, Wright C, Ballou R, Hollingdale M. 1992. A protective monoclonal antibody with dual specificity for *Plasmodium falciparum* and *Plasmodium berghei* circumsporozoite proteins. Exp Parasitol 74(4):431-440.

ANIMAL MODELS

I. Infection of monkeys with *Plasmodium* spp.

by **William E. Collins**

Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 4770 Buford Highway, Chamblee, GA 30341, USA
e-mail: wec1@cdc.gov

Equipment

compound microscope
centrifuge
water bath (37 °C)
liquid N₂ freezer
Neubauer chamber
Monolet

Materials and reagents

syringes
16g, 19g, 21g, and 27g needles
25 × 75-mm microscope slides
cryovials (Nunc)
Glycerolyte 57 (Baxter Health Care - Fenwall Division)
Giemsa stain
heparinized vacutainer tubes
phosphate buffer (pH 7.0)
RPMI 1640
50-mL centrifuge tubes
12% NaCl
1.6% NaCl
0.9% NaCl + 2% dextrose solution

Preparations

Infection with fresh parasitized erythrocytes

- Collect blood from donor animal (using femoral vein) into a heparinized tube.
- Based on the parasite count, aseptically dilute blood in RPMI 1640 to give the required number of parasites in 1 mL or less.
- Restrain (New World) or anesthetize (larger Old World) monkeys.
- Inject blood slowly into femoral vein using a 25 g needle; maintain pressure on the needle exit hole until bleeding stops.

Infection with frozen parasitized erythrocytes

- Thaw vial(s) of frozen blood rapidly in 37 °C water bath.
- Rapidly transfer cells to a 50-mL tube by sterile pipette (note volume).
- Poke a hole in the cap of the 50-mL tube with a 16g needle.
- Draw 0.2× blood volume of 12% NaCl into a syringe and 21g needle.
- Add 12% NaCl dropwise through the hole while shaking the tube.
- Let the tube stand at room temp for 5 min without shaking.
- Take up 10× volume (of cells) of 1.6% NaCl.
- Add the NaCl solution dropwise through the hole while shaking the tube.

- Spin the tube at 1,400 rpm for 10 min.
- Remove the supernatant by aspiration and suspend the pellet by gentle shaking into 10× cell volume of 0.9% NaCl and 2% dextrose solution as above.
- Spin at 1,400 rpm for 10 min.
- Remove the supernatant by aspiration and suspend the pellet by gentle shaking into 1 mL of RPMI 1640.
- Inoculate recipient monkey via the femoral vein using a syringe with 25g needle.

Comment

Viable parasites have been stored frozen for >25 years; thawing and refreezing has markedly reduced their viability.

Infection via mosquito bite

- Cage infected mosquitoes in a small container covered with nylon netting.
- Anesthetize and immobilize the monkey on a restraining board using Velcro straps (the center of the restraining board is cut out to allow the belly of the monkey to rest directly on top of the feeding cage).
- Allow mosquitoes to feed to engorgement (usually 10 to 15 min).
- After feeding, dissect mosquito salivary glands and examine them for presence of sporozoites to determine number of infected mosquitoes.
- Determine the number of sporozoites remaining in the glands:
 - 1+ = 1 to 10 sporozoites
 - 2+ = 11 to 100 sporozoites
 - 3+ = 101 to 1,000 sporozoites
 - 4+ = >1,000 sporozoites
- Record the total number of +'s for all mosquitoes fed on the animal to determine the exposure index.

Infection with sporozoites harvested by dissection

- Kill mosquitoes by exposure to chloroform.
- Remove legs and wings.
- Transfer bodies to a clean slide (wiped with alcohol just prior to use).
- Remove salivary glands from the thorax by gently pulling the base of the head from the body using either a fine needle or a 27g needle on a 1-mL syringe into a drop of 20% heat-inactivated monkey serum/saline (homologous species).
- Cut the salivary duct and remove the body and head. Ten or more sets of glands can be harvested into one drop of serum/saline.
- Add a coverslip and apply gentle pressure to rupture glands.
- Gently remove the coverslip and wash sporozoites from coverslip onto the slide with several drops of serum/saline.
- Aspirate the sporozoite suspension into a syringe with 25g needle.
- Transfer the suspension to a Nunc vial.
- Adjust the volume to approximately 1 mL.
- Repeatedly aspirate and express the suspension from the syringe to further dissociate sporozoites from salivary gland tissue.
- Transfer a small volume to a Neubauer cell counting chamber and allow it to set for 15 min.
- Calculate the number of sporozoites available.
- Dilute the suspension to the desired number of sporozoites in 1 mL serum/saline.

- Inject into the femoral vein using a 25g needle.

Comments

Infection of New World monkeys with *P. falciparum* and *P. vivax* via sporozoites has required the injection of large numbers of sporozoites, either via the bites of many heavily infected mosquitoes or via needle-injection of dissected sporozoites. Increasing the number of sporozoites injected has resulted in greater transmission rates and shorter prepatent periods.

Monitoring parasitemia in the infected monkey

- Remove the animal from the cage and immobilize it.
- Thoroughly clean either an area of the tail or the back of the leg.
- Prick with a “Monolet”, carefully avoiding the vein.
- From the drop of blood expressed, make thick and thin blood films on a precleaned, prelabeled microscope slide. Label the slide with the animal number and date.
- After the thin film dries, fix it with methanol.
- Stain the slide with Giemsa stain for 35 to 45 min.
- Examine the slide for presence of parasites.
- Express the parasite counts as
 - 1) parasites per 10,000 RBC,
 - 2) parasites per 100 WBC, or
 - 3) parasites per μL .
- If the latter, the method of Earle and Perez is preferred. Using a 5- μL pipette, collect the blood and express it onto a 15-mm $\times$ 5-mm area of a precleaned, prelabeled microscope slide (area is usually prescribed on the slide tray under the slide). Add a second drop of blood to the same slide to make the thin film.

Freezing parasitized erythrocytes

- Collect blood from the femoral vein into a heparinized tube.
- Spin the tube for 10 min at 1,200 rpm.
- Estimate the packed cell volume.
- Remove the plasma.
- Wash the cells 2 times with RPMI.
- Calculate $2\times$ RBC volume (volume of Glycerolyte needed).
- Dropwise add one-fifth of the Glycerolyte volume to the tube at 1 or 2 drops per second with shaking, then let the tube stand for 5 min.
- Add the remaining Glycerolyte dropwise at same rate with shaking.
- Aliquot 0.5 mL to 1.0 mL per labeled Nunc vial.
- Transfer the vials to a -70°C freezer overnight; transfer them to a vapor phase liquid nitrogen freezer for storage.

References

Earle WC, Perez M. 1932. Enumeration of parasites in the blood of malarial patients. *J Lab Clin Med* 17:1146-1168.

Sullivan JS, Strobert E, Yang C, Morris CL, Galland GG, Richardson BB, Bounngaseng A, Kendall J, McClure H, Collins WE. 2001. Adaptation of a strain of *Plasmodium vivax* from India to New World monkeys, chimpanzees, and anopheline mosquitoes. *J Parasitol* 87(6):1398-1403.

II. Infection of mosquitoes with *Plasmodium* spp. in monkeys

by **William E. Collins**

Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 4770 Buford Highway, Chamblee, Georgia 30341 USA

e-mail: wec1@cdc.gov

Equipment

enamelware or plastic pans
incubator (25 °C)

Materials and reagents

deionized water
baker's yeast
mosquito larval food
monkey/rat chow
lactalbumen (Sigma L-7252)
brewer's yeast (Sigma YBD)
10% sugar solution
cotton balls
blood source (rabbit/Guinea pig)
Parafilm

Preparation

Rearing anopheline mosquitoes

- Grind monkey or rat chow in a mill and sieve through a 60-mesh screen.
- Combine lactalbumen, inactive brewer's yeast, and chow (1:1:1).
- Store the mixture in a refrigerator.
- Allow mosquito eggs to hatch in a pan lined with strips of paper toweling.
- Feed a "pinch" of active baker's yeast on *Day 1*.
- On succeeding days, feed increasing amounts of mosquito larval food.
- Maintain the water depth at 2 to 3 cm.
- Every 3 or 4 days, split pans until *Day 8* to give approximately 1 larva/cm² of water surface.
- When pupae appear, strain and suspend larvae and pupae in ice water.
- Pour floating pupae into an emergence cup.
- Return the larvae to the rearing pan.
- Place the pupae in a cage for emergence.
- After they emerge, feed them a 10% sugar solution daily on a cotton ball.
- Beginning 3 days after emergence, offer them a blood meal from an anesthetized rabbit or guinea pig every 3 or 4 days. (Shave hair from the feeding area.).
- Three days after each blood meal, put a small bowl with water (1 to 2 cm depth) into the cage overnight to collect eggs.

Comments

Most commonly reared anopheline mosquitoes such as *Anopheles stephensi*, *A. atroparvus*, *A. quadrimaculatus*, *A. gambiae*, and *A. albimanus* will mate in cages; others such as *A. dirus* require force mating. The most suitable vector depends on the species and strain of *Plasmodium* being investigated.

Infection of mosquitoes by feeding on monkey

- Transfer female mosquitoes (3 to 7 days after emergence) to infection cages.
- Concentrate female mosquitoes by placing a warm hand to the side of the holding cage.
- Aspirate and transfer the mosquitoes to the infection cage.
- Partially starve mosquitoes overnight with 5% sugar solution.
- Anesthetize and immobilize the monkey on a restraining board using Velcro straps. (The center of the restraining board is cut out to allow the belly of the monkey to rest directly on top of the feeding cage.)
- Allow mosquitoes to feed to repletion (usually 10 to 15 min); remove and discard unfed mosquitoes.
- Store cartons of mosquitoes in a 25 °C incubator.
- Feed mosquitoes a 10% sugar solution during extrinsic incubation.
- Seven to 10 days after feeding (depending on the species of *Plasmodium*), dissect aliquots of mosquitoes to determine presence and numbers of oocysts.
- If oocysts are present, return undissected mosquitoes to the incubator.
- Dissect and examine salivary glands 10 to 18 days after feeding (depending on the temperature and species of *Plasmodium*).

Comments

Development of oocysts depends on the temperature and the species of *Plasmodium*. When incubated at 25 °C, the quotidian parasite *P. knowlesi* completes sporogonic development in 10 days; the tertian parasites *P. vivax*, *P. ovale*, *P. cynomolgi*, *P. gonderi*, *P. coatneyi*, *P. fragile*, *P. fieldi*, *P. simium*, and *P. simiovale* in 10 to 15 days; the quartan parasites *P. malariae*, *P. brasilianum*, and *P. inui* in 15 to 20 days.

Infection of mosquitoes by membrane feeding

- Transfer mosquitoes to infection cage (as above).
- Collect blood in heparin from the donor animal.
- Centrifuge blood at 1,500 rpm for 10 min.
- Remove plasma and replace it with serum or heparinized blood from an unexposed animal or human.
- Cover the feeding surface of a water-jacketed feeding bell with stretched Parafilm.
- Place the blood in the chamber.
- Commence circulation of water (37 °C).
- Lower the bell onto the netting of the feeding cage and allow mosquitoes to feed to repletion for 5 to 10 min.
- Remove and discard unfed mosquitoes.
- Feed mosquitoes a 10% sugar solution during extrinsic incubation.

Comments

Blood is collected in heparin, ACD, or is defibrinated with glass beads; EDTA inhibits infection. Transmission-blocking immunity often develops rapidly in monkeys. Removal of plasma and its replacement with serum or blood from an uninfected host allows continued mosquito infection if infectious gametocytes are present. Membrane feeding provides a means of assessing the transmission-blocking activity of immune sera by combining dilutions of sera from immunized

monkeys with infectious gametocytes. Reduction in oocyst densities measures transmission-blocking activity.

References

Earle WC, Perez M. 1932. Enumeration of parasites in the blood of malarial patients. J Lab Clin Med 17:1146-1168.

Rutledge LC, Ward RA, Gould DJ. 1964. Studies on feeding response to nutritive solutions in a new membrane feeder. Mosq News 24:407-409.

Sullivan JS, Strobert E, Yang C, Morris CL, Galland GG, Richardson BB, Bounngaseng A, Kendall J, McClure H, Collins WE. 2001. Adaptation of a strain of *Plasmodium vivax* from India to New World monkeys, chimpanzees, and anopheline mosquitoes. J Parasitol 87(6):1398-1403.

III. Culturing erythrocytic stages of *Plasmodium chabaudi*: a mouse malaria model

by **Masashi Hayano**^{1,2} and **Helena Helmby**³

¹Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

²Department of Tropical Medicine, Jikei University, Tokyo, Japan

e-mail: mhayano@imun.su.se

³Immunology Unit, Department of Infectious and Tropical Diseases, The London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK

e-mail: helena.helmby@lshtm.ac.uk

Equipment

hemocytometer

Materials and reagents

0.05% *p*-aminobenzoic acid (PABA, Sigma)

Giemsa stain (Gurr's improved Giemsa, BDH Laboratory Supplies)

acridine orange (AO) (optional)

heparinized tubes

methanol

Choice of mouse strain

C57Bl/6-resistant mice (clear the parasitemia and become immune)

BALB/c-semisusceptible mice (some die, survivors become immune)

A/J 100%-susceptible mice (all die)

For optimal parasite growth, add *p*-aminobenzoic acid to the drinking water of the mice.

Frozen stabilates

Bleed mice with high parasitemia (>20%) into heparinized tubes.

Aliquot blood (100 μ L/tube) in cryotubes and freeze immediately in liquid nitrogen.

(For short periods, the tubes can be frozen and stored at -70°C .)

Since parasite virulence can increase after a number of in vivo passages, it is important to start from a fresh stock of stabilates when mortality starts to increase. (Mortality is best observed in a semisusceptible strain.) Freeze new stabilates regularly.

To start infection

- Thaw a cryotube of parasites.
- Inject undiluted contents immediately i.p. into a naive recipient mouse.
- After 7 to 14 days, parasites appear in the blood.

Counting parasitemia

- Make a thin blood film from tail blood, fix with methanol for 2 min, and stain with Giemsa.
- Count percent infected cells (100 $\times$ oil immersion).
Instead of Giemsa staining, acridine orange (AO) staining of thin blood films is possible. After methanol fixing, add a small drop of AO on a slide, add a

coverslip, and count parasitemia under a light and UV-light microscope (see PARASITES, sections V:A, C).

Passage of infection into new mice

- Bleed the donor mouse.
- Count parasitemia as above and the number of erythrocytes per microliter in a hemocytometer.
- Dilute the blood in sterile PBS to 10^7 infected erythrocytes per milliliter.
- Inject 100 μ L i.p. per mouse (10^6 infected erythrocytes per dose).
- Parasites appear after 2 to 3 days, and after 8 to 10 days the parasitemia is around 30-50%.
- Mice succumbing to the infection usually die on *Days 9-12*.

Higher infectious doses give an earlier peak of parasitemia, while lower doses give a delayed peak; i.e., a 10 $\times$ concentrated dose gives a peak approximately 1 day earlier, while a dose diluted 1:10 causes a peak 1 day later.

If you inject i.v., only one-tenth of the i.p. dose is needed to obtain similar parasitemias.

Reference

Cox FEG. 1988. Major animal models in malaria research. *In*: Wernsdorfer WH, McGregor I, eds. *Malaria: principles and practice of malariology*. Vol. 2. Edinburgh: Churchill Livingstone; pp. 1503-1543

IMMUNOCHEMISTRY

I. Studies of the *Plasmodium falciparum*-infected erythrocyte surface

I:A. Surface iodination of PRBC (Lactoperoxidase method)

by **Victor Fernandez**

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: victor.fernandez@smi.ki.se

Materials and reagents

parasite culture at 5% hematocrit and >5% parasitemia

^{125}I -Na 1 mCi/10 μL (Amersham IMS.30)

PBS (pH 7.2) (stored at 4 °C)

lactoperoxidase, 2 mg/mL (Sigma L8257) (stored at -20 °C)

30% H_2O_2 (stored at 4 °C)

labelling buffer:

100 mL PBS (pH 7.2)

0.087 g K_2HPO_4

0.1 mL 1 mM KI

washing buffer:

100 mL PBS (pH 7.2)

0.83 g KI

Procedure (On ice at all times)

- Use cells from a culture of 10 to 20% parasitemia (not lower than 5% at any case) with a majority of trophozoite stages.
- Wash the cells 3 times with PBS, then transfer 200 μL of the washed packed PRBC into a fresh centrifuge tube and add 800 μL of labelling buffer.
- Add 0.5 to 1 mCi of ^{125}I -Na to the cell suspension.
- Add 100 μL of lactoperoxidase stock solution (2 mg/mL).
- Dilute the stock solution of H_2O_2 1,000-fold in PBS just before use.
- Add 25 μL of the diluted H_2O_2 to the cells; mix gently.
- Wait 1 min and repeat the addition 3 times (4 times altogether).
- At 1 min after the last addition of diluted H_2O_2 , wash the cells 4 times in ice-cold washing buffer.
- The cells are now ready for separation/enrichment of mature stages in Percoll/sorbitol gradients as described in PARASITES, section IV:D.

Reference

Fernandez V, Treutiger CJ, Nash GB, Wahlgren M. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. Infect Immun 66(6):2969-2975.

I:B. Solubilization/extraction of surface-radiolabelled proteins**by Victor Fernandez**

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: victor.fernandez@smi.ki.se

Equipment

microfuge, refrigerated

Materials and reagents

Solution containing:

1% Triton X-100

1 mM PMSF

1 mM EDTA

1 μ M leupeptin2 μ M pepstatin1% ϵ -amino-*n*-caproic acid in PBS

SDS–PAGE sample buffer:

2% SDS

5% 2-mercaptoethanol

Procedure

- Use the pellet of labelled PRBC enriched for mature stages on Percoll/sorbitol gradient as described above (see PARASITES, section IV:D).
- Resuspend the pellet in 0.25 to 0.5 mL of a solution containing 1% Triton X-100, 1 mM PMSF, 1 mM EDTA, 1 μ M leupeptin, 2 μ M pepstatin, and 1% ϵ -amino-*n*-caproic acid in PBS. Incubate the pellet on ice for 1 h with periodic vortexing.
- Centrifuge the samples in a microfuge at 4 °C, at maximum speed for 15 min. Separate and save the supernatant (the fraction soluble in Triton X-100).
- Resuspend/extract the pellet in SDS–PAGE sample buffer, mix well by pipetting, boil for 4 min, mix again, and centrifuge it for 3 min at maximum speed in a microfuge.
- Separate the supernatant (the fraction soluble in SDS).
- Run aliquots of Triton X-100 and SDS-soluble fractions on SDS–PAGE gels (6% homogeneous or 5 to 8.5% gradients for proteins above Band 3; i.e., M.W. ~100,000, 7.5 to 17.5% gradients for proteins below Band 3).

Reference

Fernandez V, Treutiger CJ, Nash GB, Wahlgren M. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. *Infect Immun* 66(6):2969-2975.

I:C. Alternative solubilization protocol

by **Denise Mattei** and **Artur Scherf**

Institut Pasteur, Unité BIHP, rue du Dr. Roux, 75015 Paris, France

e-mail: dmm@pasteur.fr, ascherf@pasteur.fr

Equipment

SDS–PAGE gel apparatus
microfuge

Materials and reagents

2% SDS

PBS containing 0.1 mg/mL of leupeptin and 0.1 mg/mL of chymostatin protease inhibitors

PBS containing 1% Triton X-100, 1 mM PMSF and 1% ϵ -amino-*n*-caproic acid
protein A–Sepharose (Amersham Biosciences 17-0780-01 or Sigma)

Preparation

- Solubilize ~200 μ L of erythrocytes (the pellet from one flask) in 4 to 8 mL of 2% SDS (dissolved in PBS containing 0.1 mg/mL of leupeptin and 0.1 mg/mL of chymostatin protease inhibitors).
- Incubate the sample at room temperature for 30 to 90 min. Any possible background radiation from the protein A–Sepharose can be adsorbed by mixing washed protein A–Sepharose with the solubilized sample and letting it incubate for 1 h prior to the immunoprecipitation.

Solubilization

- Extract surface-radioiodinated proteins by using 0.2 mL of pellet plus 1.8 mL of PBS containing 1% Triton X-100, 1 mM PMSF, and 1% ϵ -amino-*n*-caproic acid and incubating the tube at 4 °C on ice for 1 h.
- Centrifuge the extract in a microfuge at maximum speed for 15 min. Remove the Triton X-100 soluble supernatant, then freeze the supernatant at –70 °C.
- Extract the insoluble pellet in SDS–PAGE sample preparation buffer.
- Separate the proteins on a 7.5 to 17.5% linear gradient SDS–PAGE gel.

Reference

Mattei D, Scherf A. 1992. The Pf332 gene of *Plasmodium falciparum* codes for a giant protein that is translocated from the parasite to the membrane of infected erythrocytes. Gene 110(1):71-79.

I:D. Immunoprecipitation of ¹²⁵I-labelled PfEMP1**by Victor Fernandez***Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden**e-mail: victor.fernandez@smi.ki.se***Equipment**

microcentrifuge
PhosphorImager

Materials and reagents

¹²⁵I-labelled SDS extract, preferably use 100,000 or more cpm per sample
¹²⁵I-labelled Triton X-100, if desired
2% Triton X-100 in PBS
hybridoma supernatant to which has been added 25 mM of HEPES (pH 7.4),
0.5% Triton X-100, 5 mM EDTA, and protease inhibitors
Protein A-sepharose (Amersham Biosciences 17-0780-01)

NETT buffer:

150 mM NaCl, MW 58.44 (8.77 g/liter)
5 mM EDTA, MW 380.2 (1.9 g/liter)
50 mM Tris-base, MW 121.1 (6.06 g/liter)
0.02% sodium azide
0.5% Triton X-100
Adjust to pH 8.1 if mouse antibody is used, otherwise use pH 7.4.

NETT with final 0.3 M NaCl

NETT with 10 mg/mL Ig-free BSA containing protease inhibitors (1 mM PMSF, 1 mM EDTA, 1 mM leupeptin, 2 mM pepstatin, and 1% ϵ -amino-*n*-caproic acid in PBS)

NETT with 10 mg/mL BSA Fraction V

secondary antibodies as appropriate

protein A–Sepharose equilibrated with NETT with Ig-free BSA without protease inhibitors, make up as a 1:1 suspension (i.e., 50 μ L of Sepharose gel : 50 μ L of suspension)

5% SDS Sample Buffer (= 2 $\times$ concentration)

Procedure**Day 1**

- Reconstitute SDS extract by adding an equal volume of 2% Triton X-100 in PBS and 20 volumes of NETT plus Ig-free BSA containing protease inhibitors.
- Aliquot the reconstituted SDS extract in microfuge tubes allowing about 100,000 cpm per tube (or more if it is available).
- Add primary antibody:
For sera, use 10 μ L/sample for monoclonal antibodies (you can go up to 20 μ L with human or *Aotus* sera if desired), but rabbit sera generate a high MW aggregate which messes up this region on the gel. Use 2 to 5 μ L of hybridoma supernatant to which has been added 25 mM of HEPES (pH 7.4), 0.5% Triton X-100, 5 mM EDTA, and protease inhibitors.
- Incubate the sample overnight at 4 °C with slow rotation/mixing.

Day 2

- Add secondary antibodies if using primary antibodies not binding protein A. Leave the tubes for 1 h at room temperature.
- Add 100 μ L of the above equilibrated protein A–Sepharose.
- Leave it at room temperature for 1 h with intermittent mixing.
- Take off the supernatant and put in ^{125} I liquid waste. Wash the gel–slurry with the following solutions and quick, ~30-s spins in a microcentrifuge on the high setting:
 - 1 mL NETT–BSA
 - 1 mL NETT
 - 1 mL NETT with 0.3 M NaCl
- Leave the slurry for 20 min to allow NaCl to equilibrate into the beads. Then wash again:
 - 1 mL NETT with 0.3 M NaCl
 - 1 mL NETT
- Take off all supernatant.
- Add 50 μ L of 2 $\times$ Sample Buffer to the pellet. Mix and freeze at -70°C until use in SDS–PAGE gel electrophoresis.

Notes on the capacity of protein A–Sepharose

According to the manufacturer's specification, the capacity is 20 mg of human-IgG per milliliter of gel or 3 to 10 mg of mouse-IgG per milliliter of gel. Taking 10 mg/mL as above, then 50 μ L of gel used for immunoprecipitation has a maximum capacity of 500 μ g of IgG. Based on the principle that it is best to use less than 20% of capacity to get complete binding, one should aim to use maximally 100 μ g of IgG.

e.g., 10 μ L of rat serum = 100 μ g of IgG. Add 50 to 100 μ g of secondary antibody IgG.

Autoradiography for the measurement of γ rays

Stain, destain, and dry the SDS–PAGE gel. Expose a PhosphorImager screen (or an X-ray film using an intensifying screen) at room temperature.

Reference

van Schravendijk MR, Rock EP, Marsh K, Ito Y, Aikawa M, Neequaye J, Ofori-Adjei D, Rodriguez R, Patarroyo ME, Howard RJ. 1991. Characterization and localization of *Plasmodium falciparum* surface antigens on infected erythrocytes from West African patients. Blood 78(1):226-236.

II. Metabolic labelling of *Plasmodium falciparum*

II:A. Metabolic labelling of *Plasmodium falciparum*-infected erythrocytes

by **Victor Fernandez**

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: victor.fernandez@smi.ki.se

Materials and reagents

RPMI custom medium, containing everything including serum, but without methionine

healthy parasites at ring-stage

0.5 mCi of ³⁵S-methionine (Amersham SJ1015)

RPMI 1640

Procedure

- Use ring-form parasites to label, then let them develop into late stages (20 to 30 h) for analysis.
- Use RPMI custom medium, containing everything including serum, but without methionine.
- Use as high a parasitemia as possible (i.e., healthy parasites at ring-stage).
- Use 0.5 mCi of ³⁵S-methionine per small culture flask (25 cm²).
- Remove the old medium, as usual, leaving a few drops of the old medium in the flask.
- Mix 0.5 mCi of ³⁵S-methionine with 5 mL of medium and add to the flask.
- Incubate the flask as usual at 37 °C for approximately 30 h, until late parasite stages develop.
- Wash the parasites 3 times with RPMI 1640.
- Freeze the parasite pellet at –20 °C.

Comments on autoradiography for measuring β rays

- Stain and destain the gel. If it will be exposed on an X-ray film, use Amplify (Amersham) after the last destaining bath. Use enough Amplify for the gel to float freely and let it soak for 15 to 20 min. This step is not needed if a PhosphorImager screen will be used.
- Dry the gel. Expose the X-ray film to the gel at –70 °C using an intensifying screen, or simply use a PhosphorImager screen at room temperature.

Reference

Helmby H, Cavelier L, Pettersson U, Wahlgren M. 1993. Rosetting *Plasmodium falciparum*-infected erythrocytes express unique strain-specific antigens on their surface. Infect Immun 61(1):284-288.

II:B. Immunoprecipitation using metabolically labelled parasite extracts*by Victor Fernandez**Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden**e-mail: victor.fernandez@smi.ki.se***Equipment**

immufuge

Materials and reagents

NETT-buffer 1 (NETT-1):

150 mM NaCl, MW 58.44 (8.77 g/L)

5 mM EDTA, MW 380.2 (1.9 g/L)

50 mM Tris-base, MW 121.1 (6.06 g/L)

0.02% sodium azide

0.5% Triton X-100

Adjust to pH 8.1 if mouse antibody is used, otherwise use pH 7.4.

NETT-buffer 2 (NETT-2):

same as above except use 500 mM NaCl (29.22 g/liter).

Adjust to pH 8.1 if mouse antibody is used, otherwise use pH 7.4.

10% Triton X-100

1% BSA

50% protein A–Sepharose (washed with NETT-buffer 1)

SDS-PAGE sample buffer

100 mM Tris-HCL, pH 6.8

10% Glycerol

5% SDS

2% 2-mercaptoethanol

bromophenol blue

0.25% Coomassie blue R-250

Amplify (Amersham)

Procedure

- Mix in a conical centrifuge tube:
 - 300 µL NETT-1
 - 100 µL 10% Triton X-100
 - 25 to 100 µL antibodies
 - 100 µL solubilized sample
 - 1% BSA
- Incubate the mix for 90 min at room temperature or 4 °C, slowly rotating/mixing.
- Add 75 µL of 50% protein A–Sepharose. Incubate the mix for 30 to 60 min at room temperature. Shake the tube a little now and then.
- Wash the Sepharose mixture and centrifuge it in a blood centrifuge (Immufuge) for 15 s on low setting:
 - twice with NETT-1
 - once with NETT-2
 - twice with NETT-1
- Add 50 µL of sample prep. buffer and boil the solution for 2 min. Run the sample on SDS–PAGE.

- Stain and destain the gel, use Amplify for no more than 20 min (see IMMUNOCHEMISTRY, section II:A above), then dry the gel at 60 °C.
- Put the dried gel on film, without intensifying screen, in a cassette at –70 °C for at least two weeks or simply use a PhosphorImager screen at room temperature.

Reference

Helmby H, Cavelier L, Pettersson U, Wahlgren M. 1993. Rosetting *Plasmodium falciparum*-infected erythrocytes express unique strain-specific antigens on their surface. Infect Immun 61(1):284-288.

III. Resolution of giant proteins (200 kDa to 1 MDa) of *Plasmodium falciparum* on polyacrylamide–agarose composite gels

by **Denise Mattei** and **Artur Scherf**

Institut Pasteur, Unité BIHP, rue du Dr. Roux, 75015 Paris, France

e-mail: dmm@pasteur.fr, ascherf@pasteur.fr

Equipment

SDS–PAGE gel apparatus

Materials and reagents

polyacrylamide–agarose composite gel:

375 mM Tris–HCl (pH 8.8)

0.5% agarose

3% acrylamide (29:1, acrylamide:bisacrylamide)

0.1% SDS

ammonium persulfate

N,N,N',N'-tetramethyl-ethylenediamine (TEMED)

sample buffer:

10% SDS

250 mM Tris–HCl (pH 8.8)

20% glycerol

0.1% bromphenol blue

running buffer:

25 mM Tris–HCl (pH 8.3)

250 mM glycine

0.1% SDS

transfer buffer:

48 mM Tris

39 mM glycine

0.0375% SDS

3% 2-mercaptoethanol (optional)

Preparation

- Prepare the polyacrylamide–agarose composite gel. Dissolve the agarose by boiling it in the Tris buffer, cool it to 50 °C, and then add the acrylamide and SDS.
- Initiate the polymerization by adding ammonium persulfate and TEMED to a final concentration of 0.1% and 1 µL/mL of gel, respectively. Cast the gel in a standard horizontal chamber without a stacking gel layer.
- Boil the protein extracts for 5 min in sample buffer and load them on the gel. The addition of 3% 2-mercaptoethanol to the sample buffer is optional. In the presence of 2-mercaptoethanol, samples are denatured by heating to 50 °C for 15 min.
- Size-fractionate the proteins at 15 V/cm in running buffer for 1 to 2 h.
- Transfer the size-fractionated proteins to a nylon membrane using a semidry transfer device at 3.5 mA/cm² in transfer buffer for 2 h.

Reference

Wiesner J, Mattei D, Scherf A, Lanzer M. 1998. Biology of giant proteins of *Plasmodium*: resolution on polyacrylamide–agarose composite gels. *Parasitol Today* 14(1):38-40.

IV. Recovery of *Plasmodium falciparum* native proteins and active enzymes

IV:A. Solubilization in the presence of non-detergent sulphobetaines, mild solubilization agents for protein purification

by **Catherine Braun-Breton**

Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur, 75724 Paris, France
e-mail: cbb@pasteur.fr

Equipment

centrifuge

Materials and reagents

RPMI 1640

10% Triton X-100 (stock solution)

mixed ion-exchange beads AG 501-X8 (Bio-Rad)

3 M NDSB 201 (3-{1-pyridinio}-1-propanesulfonate) (Fluka) dissolved in warm water and repurified on mixed ion-exchange beads; kept at -20°C

2% Triton X-100, 2 M NDSB 201 (extraction solution; kept at -20°C)

leupeptin and pepstatin (10 $\mu\text{g/mL}$) (optional) (stock solutions)

double-distilled water

sulphobetaine SB3-14 (optional)

Preparation

- Wash pelleted PRBC twice in RPMI 1640.
- Resuspend the pellet in 1 volume of double-distilled water.
- Freeze at -20°C and thaw at room temperature.
- Add 2 volumes of:
 - 2% Triton X-100 (or detergent sulphobetaine SB3-14)
 - 2 M NDSB 201
 - leupeptin and pepstatin (10 $\mu\text{g/mL}$), if needed
 - no** Tris buffer (usually 10 mM [pH 7.5]) if followed by isoelectric focusing
- Incubate the pellet for 30 min at room temperature.
- Centrifuge the pellet for 30 min at $15,000 \times g$ at 4°C ; keep the supernatant (extract) frozen at -20°C .

Reference

Vuillard L, Braun-Breton C, Rabilloud T. 1995. Non-detergent sulphobetaines: a new class of mild solubilization agents for protein purification. *Biochem J* 305(1):337-343.

IV:B. Isoelectric focusing of proteins in a Rotofor cell: a first step in protein purification**by Catherine Braun-Breton***Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur, 75724 Paris, France**e-mail: cbb@pasteur.fr*

The Rotofor cell is a preparative scale, free solution, isoelectric focusing apparatus useful in a protein purification scheme. The cell can achieve 25-fold purification of a protein in a single 4-h run. This technique is especially advantageous when protein activity must be maintained. Bioactivity is maintained because the proteins are in solution in their native conformation.

Equipment

Rotofor cell (Bio-Rad)

pH meter

Materials and reagents

0.1 M NaOH

0.1 M H₃PO₄

1% Triton X-100

1 M NDSB 201 (3-{1-pyridinio}-1-propanesulfonate) (Fluka)

2% ampholytes (Pharmalytes of the appropriate pH range, usually between pH 3 and pH 10, 40% stock solution, Pharmacia)

Preparation

- Equilibrate the ion exchange membranes by overnight incubation in the appropriate electrolyte solution: 0.1 M NaOH for the anion exchange membrane and 0.1 M H₃PO₄ for the cation exchange membrane.
- Cool the Rotofor cell by connecting the cooling finger to a source of circulating coolant.
- Assemble the focusing chamber according to the instruction manual.
- Fill the electrolyte chambers with electrolytes immediately after assembly to prevent the membranes from drying: 25 mL of 0.1 M NaOH for the cathode chamber (–) containing the anion exchange membrane and 25 mL of 0.1 M H₃PO₄ for the anode chamber (+) containing the cation exchange membrane.
- Fill the focusing chamber with 45 mL of focusing solution, using a 50-mL syringe with a needle:
 - 1% Triton X-100 (or SB3-14; not necessary for soluble proteins)
 - 1 M NDSB 201
 - 2% ampholytes
- Prerun the chamber for 1 h at 4 °C, 11 W, 34 mA, and 995 V to preform the pH gradient.
- Load 1 to 5 mL of the sample (protein solution or extract) in 1 M NDSB 201, 2% ampholytes.
- Add the sample in the central channels of the Rotofor focusing chamber to prevent exposure of the proteins to very low or high pH.
- Protein concentration should be adjusted for desired yield. Assuming each component will focus in 1 to 3 fractions; preparative fractionation of 1 to 2 g of proteins has been successfully performed!
- Run the sample for 3 h at 4 °C, 11 W, 34 mA, and 995 V.

- Collect the 20 fractions on ice.
- Measure the pH of each fraction. Take a 100- μ L aliquot, add 1 mL of double-distilled water, and keep it at room temperature for 10 min before measuring the pH.
- Store the fractions at -20°C .

Reference

Roggwiller E, Morales Bétoulle MR, Blisnick T, Braun-Breton C. 1996. A role for erythrocyte band 3 degradation by the parasite gp76 serine protease in the formation of the parasitophorous vacuole during invasion of erythrocytes by *Plasmodium falciparum*. Mol Biochem Parasitol 82(1):13-24.

SEROLOGY

I. Antibody staining of *Plasmodium falciparum*-infected erythrocytes

I:A. Erythrocyte membrane immunofluorescence (EMIF)

by **Hedvig Perlmann**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: hedvig.perlmann@imun.su.se

Equipment

centrifuge
humid chamber (wet filter paper in a plastic box)
microscope (UV and light)

Materials and reagents

Plasmodium falciparum cultures in human type O⁺ erythrocytes, 5 to 10% parasitemia (see PARASITES, section I:A)
glutaraldehyde (GDA)
biotinylated anti-Ig of the appropriate specificity (Vector Labs)
FITC–Avidine D, F/P 4.9 (Vector Labs).
ethidium bromide (Sigma)
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
Tris (Sigma)
Hanks' balanced salt solution (Gibco)
multitest slides (8 wells; ICN Biomedicals)
Pasteur pipettes
micropipettes

TH-buffer (0.15 M Tris-buffered Hanks', pH 7.2):

2.11 g Tris–HCl

0.2 g Tris-base

7.88 g of 0.9% NaCl

Dissolve the ingredients in distilled water and bring volume to 1 liter.

Mix 1 volume of Tris buffer with 1 volume of Hanks' BSS.

Add 0.02% NaN₃ (200 mg NaN₃/1 liter).

coating buffer (pH 9.6):

1.59 g Na₂CO₃

2.93 g NaHCO₃

200 mg NaN₃

Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Monolayers

- Wash cultures (5 to 10% parasitemia) in TH and resuspend them to 1% hematocrit.
- Pretreat multitest slides, 8 wells with one drop (~20 µL) per well of coating buffer for 30 min.

- Add one drop per well of the 1% *P. falciparum* culture directly after aspiration of the coating buffer and leave the slides to settle for 30 min.
- Rinse off unbound erythrocytes by gently shaking the slides immersed upside down in TH.

Glutaraldehyde (GDA)-fixation of monolayers

- Quickly but gently cover the wet monolayers with 1 to 2 mL of 1% GDA (in PBS) on top of the slide.
- Decant the GDA after about 10 s and repeat the GDA fixation once.
- Wash the slides with distilled water and air-dry them. Slides can then be stored long-term in a freezer (>10 years).

Staining

All incubations below should be done at room temperature in a humid chamber for 30 min.

- Treat the slides sequentially with one drop (~20 µL) of serial dilutions of sera containing malaria antibodies.
- Use 15 µg/mL of biotinylated anti-Ig of the appropriate specificity followed by 50 µg/mL of FITC–avidin D.
- Wash the slides between the incubations with TH or PBS.
- For counter-staining, use one drop of ethidium bromide solution (10 µg/mL) in each well and rinse the slides with distilled water after a few seconds.

Reference

Perlmann H, Berzins K, Wahlgren M, Carlsson J, Björkman A, Patarroyo ME, Perlmann P. 1984. Antibodies in malarial sera to parasite antigens in the membrane of erythrocytes infected with early asexual stages of *Plasmodium falciparum*. J Exp Med 159(6):1686-1704.

I:B. Erythrocyte membrane staining by enzyme linked antibodies (EMEAS)

by **Rachanee Udomsangpetch**

Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand e-mail: scrud@mahidol.ac.th

Prepare glutaraldehyde-fixed monolayers as in the protocol for EMIF (see SEROLOGY, section I:A).

Equipment

light microscope with a 100× oil-immersion lens
centrifuge
humid chamber (wet filter paper in a plastic box or dish)

Materials and reagents

Plasmodium falciparum cultures in human type O⁺ erythrocytes, 5 to 10% parasitemia (see PARASITES, section I:A)
multitest slides (8 wells; ICN Biomedicals)
Pasteur pipettes
micropipettes
biotinylated anti-Ig (Vector Labs)
streptavidin/ALP (Mabtech)
PBS
ALP-conjugated Substrate Kit (Bio-Rad 170-6432)

coating buffer (pH 9.6):

1.59 g Na₂CO₃

2.93 g NaHCO₃,

200 mg NaN₃

Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Staining

All incubations below should be done at room temperature in a humid chamber for 30 min.

- Treat the slides sequentially with serial dilutions of immune sera.
- Use biotinylated anti-Ig of the appropriate specificity and streptavidin/ALP.
- Wash the slides between the incubations with PBS.
- Add freshly prepared substrate.
- Wash slides under distilled water after 20 min, then air-dry them.
- Examine the slides under a light microscope with a 100× oil-immersion lens.

Reference

Udomsangpetch R, Kulane A, Berzins K, Perlmann H, Perlmann P, Wåhlin Flyg B. 1993. Enzyme-linked staining method for light microscopic detection of antibodies to parasite antigens on the membrane of *Plasmodium falciparum* infected erythrocytes. Acta Trop 55(1-2):79-86.

I:C. Parasite immunofluorescence (PARIF)

by **Rachanee Udomsangpetch**

Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand e-mail: scrud@mahidol.ac.th

This protocol allows for antibody staining of intracellular *Plasmodium falciparum* using air-dried monolayers of infected erythrocytes. For staining of the live, intact PRBC surface, please refer to PARASITES, section IX:B.

Equipment

fan

Materials and reagents

Plasmodium falciparum cultures in human type O⁺ erythrocytes, 5 to 10% parasitemia of primarily late stages
PBS

coating buffer (pH 9.6):

1.59 g Na₂CO₃

2.93 g NaHCO₃

200 mg NaN₃

Add distilled water up to 1 liter.

Monolayers

- Use *Plasmodium falciparum* cultures in human type O⁺ erythrocytes, 5 to 10% parasitemia of primarily late stages.
- Wash cells in PBS and resuspend them to 1% parasitemia.
- Pretreat multitest slides with one drop (10 to 20 µL) per well of a coating buffer for 30 min.
- Aspirate the wells and immediately add one drop of the washed culture to each well.
- Leave it to settle for 30 min.
- Gently aspirate the excess of red cells from each well and air-dry the slides under a fan.
- The slides can be stored in a freezer; dry them well before staining.
- For staining, see the protocol for EMIF in SEROLOGY, section I:A above.

Reference

Udomsangpetch R, Carlsson J, Wählin B, Holmquist G, Ozaki LS, Scherf A, Mattei D, Mercereau-Puijalon O, Uni S, Aikawa M, Berzins K, Perlmann P. 1989. Reactivity of the human monoclonal antibody 33G2 with repeated sequences of three distinct *Plasmodium falciparum* antigens. J Immunol 142(10):3620-3626.

II. Antibody selection on immobilized antigen or peptide

by **Denise Mattei**

Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur, 75724 Paris, France

e-mail: dmm@pasteur.fr

Equipment

PD10 column equilibrated in PBS

Materials and reagents

0.45- μ m nitrocellulose membrane

PBS

PBS plus 5% powdered skim milk

3 M MgCl_2 , 75 mM HEPES/NaOH (pH 7.2), 25% ethylene glycol

Preparation

- Prepare 5 mL of a 5 to 10 $\mu\text{g/mL}$ solution of peptide or protein in PBS.
- Soak a 0.45- μ m nitrocellulose-membrane filter in this solution for 30 min at room temperature with gentle agitation, and then soak a second filter in this solution.
- Incubate the filters for saturation in PBS plus 5% powdered skim milk for 90 min at room temperature (or overnight at 4 °C) with gentle agitation.
- Incubate each filter in 5 mL of a 1:20 dilution of appropriate serum in PBS plus 5% powdered skim milk for 60 min at room temperature (or overnight at 4 °C) with gentle agitation.
- Wash the filters in PBS plus 5% powdered skim milk for 30 min at room temperature with gentle agitation.
- Wash the filters twice in PBS.
- Soak the filters in 5 mL of a solution of 3 M MgCl_2 , 75 mM HEPES/NaOH (pH 7.2), 25% ethylene glycol.
- Incubate the filters for 20 min at room temperature with gentle agitation.
- Recover the solution and load it on a PD10 column equilibrated in PBS for collection of 500- μL fractions.
- Check for the presence of IgG in the fractions by dot blot analysis (5 μL of each fraction).

Reference

Tsang VCW, Wilkins PP. 1991. Optimum dissociating condition for immunoaffinity and preferential isolation of antibodies with high specific activity. *J Immunol Methods* 138(2): 291-299.

III. ELISA

III:A. Equipment, materials, and reagents

by **Hedvig Perlmann**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: hedvig.perlmann@imun.su.se

Equipment

micropipette, multichannel pipette (e.g., Thermo Electron)
microplate washer (e.g., Thermo Electron, Skatron Instruments)
microplate reader (e.g., V max kinetic microplate reader with computer program
SOFTmax Cat. No. 79-200 105, 79-200 100)
centrifuge, swing out rotor

Materials and reagents

With many proteins or peptides, PBS can be used as a coating solution at least with the above plates; test your system first.

flat-bottomed microtiter plates (Maxisorp from Nunc A/S or High Binding from
Costar, Cat. No. 3590)
disposable pipette tips
tubes for preparations of dilutions (Bio-Rad, Cat. No. 223-9391)
cell culture flasks
RPMI–HEPES (see PARASITES, section I:A)
Hanks' balanced salt solution (Gibco)

PBS 10× stock solution:

40 g $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$

5 g KH_2PO_4

81 g NaCl

1.0 mL 20% NaN_3

Dissolve in distilled water and bring volume to 1 liter.

Coating buffer (pH 9.6):

1.59 g Na_2CO_3

2.93 g NaHCO_3

1.0 mL 20% NaN_3

Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Blocking buffer:

0.5% BSA in coating buffer

Tween buffer for dilutions and incubations:

100 mL PBS stock (10×, see above)

5 g BSA

0.5 mL Tween

1.0 mL 20% NaN_3

Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Tween wash:

45 g NaCl

2.5 mL Tween

Dissolve the ingredients in distilled water and bring the volume to 5 liters.

Enzyme substrate buffer:

97 mL diethanolamine

~700 mL distilled water

1 mL 20% NaN_3 Adjust the pH to 9.8 with 1 M HCl (~100 mL), then add 101 mg of $\text{MgCl}_2 \cdot \text{H}_2\text{O}$. Bring buffer volume to 1 liter.

Substrate:

NPP (kept in freezer)

Dissolve 1 tablet of NPP in 5 mL of enzyme substrate buffer; keep the solution in the dark. Incubate plates with the substrate at room temperature and keep them in the dark until you are ready for a reading at OD_{405} (usually in 20 to 40 min).

TH (0.15 M Tris-buffered Hanks', pH 7.2):

2.11 g Tris-HCl

0.2 g Tris-base

7.88 g NaCl

Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Add 1.0 mL of 20% NaN_3 . Mix 1 volume of Tris buffer with 1 volume of Hanks' balanced salt solution.

Sera:

For antibody determinations, sera may be kept in the refrigerator diluted 1:10 in Tris-buffered Hanks' with 0.02% NaN_3 for many months.

Conjugate:

ALP-conjugated or biotinylated anti-Ig of appropriate specificity at recommended concentration

Streptavidine-ALP (Mabtech) when biotinylated antibody has been used.

60% Percoll solution:

60 mL Percoll

7 mL 10× PBS

33 mL PBS

0.3 M NH_4OH

III:B. Antigens for coating

by **Hedvig Perlmann**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: hedvig.perlmann@imun.su.se

Crude parasite antigen

Late stage-infected erythrocytes enriched by gradient centrifugation. For 40 tubes, 80-mL of culture:

- Prepare fresh 60% Percoll solutions before each use. Keep all solutions on ice throughout the preparation.
- Wash a late stage *Plasmodium falciparum* culture once in RPMI-HEPES and resuspend it to 10% hematocrit in cold RPMI-HEPES.
- Distribute in centrifuge tubes, 2 mL/tube.
- Using a Pasteur pipette, gently add 2.5 mL of cold 60% Percoll to the bottom of each tube.
- Centrifuge the tubes in a swing-out rotor at $1,500 \times g$ and 4°C for 15 min (2,000 rpm in Sorvall or Beckman centrifuge).
- Withdraw the cells at the interphases with care, pool them, and wash them 3 times with cold PBS (centrifuging each time for 7 to 8 min at 2,000 rpm). The interphases contain trophozoites and schizonts, while rings and uninfected erythrocytes are found in the pellet.
- Freeze-thaw or sonicate the isolated interphases, determine protein after centrifugation, and coat the plate at $10\text{ }\mu\text{g/mL}$ in coating buffer.
- Contaminating HGG in this antigen is avoided by using Albumax to replace serum in the medium for the parasite cultures (see PARASITES, section I:A).

Comment: 12 medium size cultivation flasks (75 cm^2 , 20 mL of culture) with late stages of 10% parasitemia give about 1.5 mg of protein in the extract.

Air-dried monolayers of human erythrocytes (of the same type as used for parasite cultures)

- Pretreat the ELISA plate with $50\text{ }\mu\text{L}$ of coating buffer for 30 min.
- Add $50\text{ }\mu\text{L}$ of freshly washed human erythrocytes (in TH or PBS) at 1% hematocrit.
- After 30 min gently and carefully rinse off any unbound erythrocytes and leave the plates to air-dry.
- Proceed as usual (see SEROLOGY, section III:C, ELISA procedure).

Peptide/BSA conjugation

- Dissolve 1 mg of peptide in 0.1 mL of distilled water.
- Add 0.3 M NH_4OH , if necessary, until the peptide is dissolved, then add PBS to a total volume of 0.5 mL.
- Dissolve 0.5 mg of BSA in the peptide solution.
- Add 0.5 mL of 0.25% glutaraldehyde in PBS dropwise while stirring.
- Mix the solution gently on a roller drum in the cold, overnight.
- Dialyse against PBS, then store at -20°C . We use $10\text{ }\mu\text{g/mL}$ for coating.

Comment: Several malaria peptides are commercially available from Bachem. Bigger peptides as MAPs can be used for coating without BSA conjugation.

III:C. ELISA procedureby **Hedvig Perlmann**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: hedvig.perlmann@imun.su.se

Procedure

- Coat the plate with 50 μ L of peptide solution or crude parasite antigen at 10 μ g/mL in coating buffer.
- Keep the plate at 4 °C overnight. With many proteins or peptides, PBS can be used as a coating solution. Test your system first.
- Block with 100 μ L of 0.5% BSA in coating buffer for 3 to 4 h at 37 °C.
- Wash 4 times with 0.9% NaCl plus 0.05% Tween.
- Add 50 μ L of serum samples diluted 1:1000; leave them for 1 h at 37 °C.
- Wash them 4 times with 0.9% NaCl plus 0.05% Tween.
- Add 50 μ L of ALP-conjugated or biotinylated anti-Ig of appropriate specificity at the recommended concentration in Tween-buffer; leave for 1 h at 37 °C.
- Wash the sample 4 times with 0.9% NaCl plus 0.05% Tween.
- If biotinylated antibody has been used, add 50 μ L of streptavidin–ALP diluted 1:2000 in Tween-buffer; leave the sample for 1 h at 37 °C.
- Wash the sample 4 times with 0.9% NaCl plus 0.05% Tween.
- Develop the sample with 50 μ L of NPP (1 tablet/5 mL of substrate buffer) (see SEROLOGY, section III:A) under Materials and reagents and read at OD₄₀₅.

References

Paulie S, Perlmann P, Perlmann H. 2003. Enzyme-linked immunosorbent assay. *In*: Celis JE, ed. Cell biology: a laboratory handbook. 3rd edition. San Diego: Elsevier Science.

Perlmann H, Perlmann P, Berzins K, Wåhlin B, Troye-Blomberg M, Hagstedt M, Andersson I, Högh B, Petersen E, Björkman A. 1989. Dissection of the human antibody response to the malaria antigen Pf155/RESA into epitope specific components. *Immunol Rev* 112:115-132.

Rougon G, Ceard B, Van Rietschoten J, Jordan B, Barbet J. 1984. Induction with a synthetic peptide of antibodies to HLA class I C-terminal intracytoplasmic region. *Mol Immunol* 21(6):461-468.

Wahlgren M, Berzins K, Perlmann P, Björkman A. 1983. Characterization of the humoral immune response in *Plasmodium falciparum* malaria. I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of microELISA. *Clin Exp Immunol* 54(1): 127-134.

CELLULAR IMMUNOLOGY

I. Preparation of human peripheral blood mononuclear cells (PBMC)

by **Marita Troye-Blomberg** and **Jacob Minang**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: marita@imun.su.se, jtminang@imun.su.se

Equipment

centrifuge (swing out rotor)
Burker chamber
light microscope

Materials and reagents

heparinized tubes (CPT sodium heparin blood collection tubes, BD Vacutainer Systems)

Pasteur pipettes

conical centrifuge tubes

Ficoll-Paque (Pharmacia)

RPMI 1640 with 20 mM HEPES (Gibco)

L-glutamine (Gibco)

penicillin–streptomycin combination (PEST) (Gibco)

fetal bovine serum (FBS) (Gibco)

tissue culture medium (TCM)

RPMI 1640–HEPES

2 mM L-glutamine

penicillin–streptomycin combination (final concentration of 100 units/mL of penicillin G sodium and 100 µg/mL of streptomycin sulfate)

10% heat-inactivated FBS

Turk gentian violet staining solution

40 mg gentian violet

12.5 mL acetic acid

Make up to 200 mL with distilled water.

Procedure

- Collect venous blood into heparinized 10-mL tubes.
- Save and freeze a small fraction of the plasma in Eppendorf tubes (1 to 2 mL for each tube) for antibody determinations.
- Mix blood in a 1:1 ratio with RPMI 1640 containing 20 mM HEPES solution and carefully layer 7 mL of the resultant suspension on top of 3 mL of Ficoll-Paque in 10-mL conical centrifuge tubes.
- Centrifuge the tubes at 2,800 rpm for 20 min. Collect the interphase with a Pasteur pipette.
- Pool and dilute ($\approx$ 10 times) the cells in RPMI 1640 containing 20 mM HEPES.
- Wash the cells 2 times, first at 1,800 rpm for 10 min and then at 1,200 rpm for 7 min and resuspend the cells in each 10-mL tube in 3 to 5 mL of TCM depending on the density/size of the pellet.

- Pipette 10 μL of the resultant cell suspension in TCM into 40 μL of TURK for counting of the lymphocytes in a Burker-chamber.

Alternative preparation of whole blood cells for cultures

- Collect 10 mL of venous blood as above.
- Pipette 10 μL of cell suspension into 30 μL of Turk for counting of the lymphocytes.
- For the cultures, dilute the blood 2, 5, or 10 times in TCM.

Reference

Smedman L, Joki A, da Silva APJ, Troye-Blomberg M, Aronsson B, Perlmann P. 1994. Immunosuppression after measles vaccination. *Acta Paediatr* 83(2):164-168.

II. Antigen preparations and peptides for cellular work

II:A. Crude parasite antigen (Percoll-band)

by **Marita Troye-Blomberg**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: marita@imun.su.se

For working with parasites and Percoll, please refer also to PARASITES and for crude antigen, please refer to SEROLOGY, section III, ELISA.

Equipment

centrifuge (swing out rotor)
sonicator
freezer at -70°C

Materials and reagents

RPMI 1640–HEPES
PBS
conical centrifuge tubes
Pasteur pipettes
0.45- μm filters (Millipore)

60% Percoll:
60 mL Percoll
7 mL 10 $\times$ PBS
33 mL 1 $\times$ PBS

Procedure

- Prepare parasite extracts from late stage cultures. Wash them once in RPMI 1640–HEPES and suspend them to 10% hematocrit in cold RPMI 1640–HEPES.
- Layer 2 mL of extract gently on top of 2.5 mL of cold 60% Percoll in 10-mL conical centrifuge tubes.
- Centrifuge the tubes at $1,500 \times g$ for 15 min at 4°C . Collect the interphase with a Pasteur pipette, pool and wash the cells 3 times with cold PBS ($1,500 \times g$ for 7 to 8 min), and resuspend them in a small volume of PBS. The interphase contains trophozoites and schizonts, while rings and uninfected erythrocytes are found in the pellet.
- Sonicate (25w) the cells with short intervals for 2 min, determine protein content, and dilute to about 2 mg/mL.
- Centrifuge the sample at $1,500 \times g$ for 10 min, filter-sterilize it, make a final protein determination, then store it in aliquots of 1 mg/mL in a -70°C freezer.

Reference

Troye-Blomberg M, Perlmann H, Patarroyo ME, Perlmann P. 1983. Regulation of the immune response in *Plasmodium falciparum* malaria II. Antigen specific proliferative responses in vitro. Clin Exp Immunol 53(2):345-353.

II:B. Desalting of synthetic peptides**by Marita Troye-Blomberg**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: marita@imun.su.se

Equipment

speed-vac

Materials and reagentsSep-Pak C₁₈ cartridge (Waters)

5-mL syringe

distilled water

methanol

Procedure

- Use a Sep-Pak C₁₈ cartridge connected with a 5-mL syringe to desalt peptides. Prewet the cartridge 3 times with 1.5 mL of methanol and wash it 2 times with 5 mL of water before use.
- Dissolve 10 mg of peptide in 10 mL of distilled water (or 10% methanol in distilled water) and pass the solution 3 times through the cartridge.
- Wash out the salts 2 times with 5 mL of distilled water, elute the peptide 2 times with 1.5 mL of 60% methanol and 2 times with 1.5 mL of 100% methanol.
- Pool the eluates, evaporate them in a speed-vac, weigh them, dissolve them in distilled water at 1 mg/mL, freeze them, and store them frozen.

Reference

Troye-Blomberg M, Riley EM, Perlmann H, Andersson G, Larsson A, Snow RW, Allen SJ, Houghten RA, Olerup O, Greenwood BM, Perlmann P. 1989. T and B cell responses of *Plasmodium falciparum* malaria-immune individuals to synthetic peptides corresponding to sequences in different regions of the *P. falciparum* antigen Pf155/RESA. J Immunol 143(9):3043-3048.

III. T-cell proliferation

by **Marita Troye-Blomberg**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: marita@imun.su.se

Equipment

incubator (37 °C, 5% CO₂ in a humid atmosphere)
scintillation counter
cell harvester (TomTec Cell Harvester)

Materials and reagents

round-bottomed 96-well microplates (Costar 3799, Corning)
(6-³H)-thymidine (specific activity 2 Ci/mmol; Amersham)
TCM (tissue culture medium), see CELLULAR IMMUNOLOGY, section I
peripheral blood mononuclear cells (PBMC)

Procedure

- Dilute PBMC in TCM to 1×10^6 cells/mL and seed 1×10^5 cells/well in 96-well round-bottomed microplates in triplicates per test.
- Add antigens (parasite extracts or recombinant proteins) or peptides at 2 to 10 µg/mL representing epitopes of interest at the initiation of the culture.
- Incubate the plates for 5 days at 37 °C in an atmosphere of 5% CO₂.
- Harvest 100 µL for cytokine determination at the time of pulsing, and then pulse with 1 µCi [6-³H]-thymidine in 100 µL of TCM for 18 h.
- Harvest the cells and measure the thymidine incorporation by liquid scintillation.
- Results are expressed as stimulation index (SI), defined as mean cpm of test triplicates divided by mean of cpm of control triplicates. An SI ≥ 2.5 is considered positive.

Reference

Troye-Blomberg M, Riley EM, Perlmann H, Andersson G, Larsson Å, Snow RW, Allen SJ, Houghten RA, Olerup O, Greenwood BM, Perlmann P. 1989. T- and B-cell responses of *Plasmodium falciparum* malaria-immune individuals to synthetic peptides corresponding to sequences in different regions of the *P. falciparum* antigen Pf155/RESA. J Immunol 143(9):3043-3048.

IV. Interleukin ELISPOT

by **Gehad El Ghazali**

Department of Clinical Immunology, Uppsala University Hospital, SE-751 85 Uppsala, Sweden

e-mail: gehad.elghazali@klinimm.uu.se

The following description should be considered as a guideline on how to stimulate and analyse cells for IFN- γ production by the ELISPOT technique. Conditions for cell preparation, stimulation, and the ELISPOT may be changed to fit with the test situation.

Equipment

incubator (37 °C, 5% CO₂ in a humid atmosphere)
multi-channel pipette
dissection microscope (40 $\times$)

Materials and reagents

nitrocellulose plates (Millipore Corp.)
mAb anti-human IFN- γ , 7-B6-1-Biotin (Mabtech)
biotinylated mAb anti-human IFN- γ , 7-B6-1-Biotin (Mabtech)
streptavidin–alkaline phosphatase (Mabtech)
BCIP/NBT substrate (Bio-Rad Laboratories)
TCM (tissue culture medium), see CELLULAR IMMUNOLOGY, section I
5-mL round-bottomed tissue culture tubes (Falcon 2058, BD Labware)
PHA (phytohemagglutinin)
PBS (filter-sterilized phosphate buffer saline) (pH 7.2)
FCS (fetal calf serum)

Procedure

Stimulation of cells can be carried out directly in the ELISPOT plate, but better results may be obtained if cells are first stimulated for a shorter period in culture tubes. In the following description cells are incubated for 2 to 4 h in tubes and thereafter transferred to ELISPOT plates for further incubation.

- Prepare sterile PBMCs.
- Count cells and suspend them in TCM to a concentration of 2×10^6 cells/mL.
- Transfer 0.5 to 1 mL of cell suspension to 5-mL round-bottomed tissue culture tubes.
- Add antigens (parasite extracts or recombinant proteins) or peptides representing epitopes of interest at a concentration of 2 μ g/mL at the initiation of the culture. As a positive control, add phytohemagglutinin (PHA) at a concentration of 5 μ g/mL.
- Incubate cells at 37 °C for 2 to 4 h.

Coating of ELISPOT plates

- Dilute the coating antibody (mAb anti-human IFN- γ , 7-B6-1-Biotin) to 15 μ g/mL in filter-sterilized phosphate buffer saline (PBS).
- Add 100 μ L/well to the nitrocellulose plates and incubate them overnight at 4 °C.

Transfer of cells to the nitrocellulose plate (sterile)

Before transferring stimulated cells to the coated ELISPOT plates, wash them 4 times with sterile PBS (200 μ L) to remove unbound coating antibody.

- Add stimulated cells to the plates. For IFN- γ , a cell concentration of 20,000 to 50,000 cells/well is usually suitable. If the number of cells exceeds 250,000 cells/well, this may result in blurry spots due to multiple cell layers (especially in the wells with cells stimulated with PHA).
- Incubate cells for 10 to 40 h at 37 °C in a humid atmosphere.

Addition of the secondary antibody (nonsterile)

- Remove cells by washing them 6 times (200 μ L/well) using PBS (multi-channel pipette).
- Dilute biotinylated mAb (anti-human IFN- γ 7-B6-1-Biotin) to 1 μ g/mL in PBS with 0.5% fetal calf serum (PBS-0.5% FCS) and add 100 μ L/well.
- Incubate the plates for 2 to 4 h at room temperature.
- Wash them in PBS 6 times (200 μ L/well).
- Add 100 μ L of streptavidin–alkaline phosphatase (diluted 1:1000 in filtered PBS-0.5% FCS) and incubate the plates for 1 to 2 h at room temperature.
- After washing them in PBS 6 times (200 μ L/well), add 100 μ L of substrate and incubate them until spots emerge (may be up to 1 h).
- Stop color development by washing the plates under running tap water (3 times at 200 μ L/well).
- Leave the plate to dry; inspect and count the spots with a dissection microscope (40 $\times$).

One-Step ELISpot kits:

Very sensitive and easy to use kits are available at Mabtech AB.

Reference

Elghazali G, Esposito F, Troye-Blomberg M. 1995. Comparison of the number of IL-4 and IFN- γ secreting cells in response to the malaria vaccine candidate antigen Pf155/RESA in two groups of naturally primed individuals living in a malaria endemic area in Burkina Faso. Scand J Immunol 42(1):39-45.

V. Reverse transcription PCR for detection of cytokines

by **Ankie Söderlund**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: ankieso@hotmail.com

Equipment

–70 °C freezer
incubator (37 °C, 5% CO₂ in a humid atmosphere)
RT-PCR kit with recombinant *AmpliTaq* DNA polymerase (Applied Biosystems)
PCR using cytokine specific primers

Materials and reagents

Ficoll-Paque (Amersham Biosciences)
round-bottomed tubes
cryotubes
liquid nitrogen
PBS
acid guanidinium thiocyanate–phenol–chloroform
DEPC-treated distilled water

Procedure

- Isolate PBMC from venous blood by Ficoll-Paque centrifugation as described in CELLULAR IMMUNOLOGY, section I.
- To detect any spontaneous cytokine production in the controls, wash unstimulated PBMC 4 times in PBS. Before the last wash, transfer cells to a cryotube, centrifuge them at $300 \times g$, and carefully remove all remaining PBS. Immediately freeze the cells in liquid nitrogen.
- To detect cytokines after antigen stimulation, place cells in round-bottomed tubes, one (or two) for each time point, and add the desired antigen. Place the tubes at 37 °C, 5% CO₂, for 4, 7, and 22 h.
- Wash the cells 4 times in PBS as described above, remove the supernatant, and freeze the cells in liquid nitrogen.
- For total RNA extraction, either use commercial kits available or the acid guanidinium thiocyanate–phenol–chloroform method by Chomczynski and Sacchi, 1987 (see also MOLECULAR BIOLOGY, section II).
- Dilute the RNA in 50 µL of DEPC-treated distilled water and thereafter keep it stored at –70 °C.
- Use an RT-PCR kit with recombinant *AmpliTaq* DNA polymerase for reverse transcription and subsequent PCR using cytokine specific primers.

References

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 162(1):156-159.

Söderlund A, Gabrielsson S, Paulie S, Hammarström ML, Rak S, Troye-Blomberg M. 1997. Allergen induced cytokine profiles in type I allergic individuals before and after immunotherapy. *Immunol Lett* 57(1-3):177-181.

VI. Detection of cytokine-gene polymorphism

VI:A. Extraction of human DNA from peripheral blood or buffy coat

by **Ben Gyan**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: bgyan@noguchi.mimcom.net

Equipment

incubator
centrifuge
gel electrophoresis apparatus
speed-vac
gel drier or oven
spectrophotometer
camera
automated thermal cycler
microscope with UV light

Materials and reagents

lysing buffer:

1.6 M sucrose
50 mM Tris-HCl (pH 7.5)
25 mM MgCl₂
5% Triton X-100

DNA buffer:

10 mL 1 M Tris-HCl (pH 7.5)
2.5 mL 0.5 M NaCl
2.5 mL 0.5 M EDTA
1.25 mL 20% SDS
Adjust volume to 50 mL using double distilled H₂O.

sterile vacutainers with EDTA (ethylamine tetra-acetic acid)
Falcon tubes, 50-mL
proteinase K solution (Invitrogen 25530 049)
20% SDS
sterile double-distilled H₂O
Pasteur pipettes
Eppendorf tubes
phenol
chloroform
3 M sodium acetate
absolute ethanol
70% ethanol

Procedure

- Draw 1 to 8 mL of venous blood into sterile vacutainers containing EDTA and keep it at room temperature (or frozen).
- Transfer blood into a 50-mL Falcon tube and add 10 to 40 mL of lysing buffer.
- Turn the tube gently upside down for 2 to 3 min.

- Centrifuge the tube at 2,000 rpm for 10 min (with brake off) and discard the supernatant.
- Add 40 μ L of proteinase K solution, 40 μ L of 20% SDS, 180 μ L of DNA buffer, and 340 μ L of sterile double-distilled H₂O.
- Mix the sample well by gently pipetting it up and down until it becomes gelatine-like.
- Digest the blood by incubating it overnight at 37 to 42 °C.
- Transfer the sample into Eppendorf tube and add 600 μ L of phenol.
- Extract the DNA by revolving/mixing the tubes until the solution becomes milky.
- Centrifuge the tube at 13,000 rpm for 10 min at room temperature.
- Transfer the upper aqueous phase (DNA) into a new Eppendorf tube.
- Add 800 μ L of chloroform.
- Extract the DNA and centrifuge the tube at 6,000 rpm for 10 min.
- Transfer the upper phase into a new Eppendorf tube.
- Precipitate the DNA by adding one-tenth volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol; allow the tube to sit for 2 hours or overnight at –20 °C. The DNA can be stored this way as well.
- Centrifuge the precipitate for 30 min at 4 °C, wash it gently once with 70% ethanol, dry it in a speed-vac, and gently resuspend the pellet in 25 to 100 μ L of distilled water, depending on its size.
- Determine the DNA concentration at OD₂₆₀; 2 mL of culture may yield ~2 μ g of genomic DNA. Visualize ~0.5 μ g on a 0.8% agarose gel to see that the DNA runs as a high-molecular weight, somewhat broad band and is thus unsheared and free of RNA.

VI:B. PCR for amplification of nucleotide repeat (VNTR) polymorphisms of cytokine genes

by **Ben Gyan**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: bgyan@noguchi.mimcom.net

Equipment

automated thermal cycler
UV-transilluminator
camera

Materials and reagents

dNTPs
primers
Taq polymerase
PCR buffer with MgCl₂
sterile H₂O
Taq gold (Applied Biosystems)
agarose gel
0.1% ethidium bromide
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

TE buffer (pH 8.0):

10 mM Tris Cl (pH 8.0)
1 mM EDTA (pH 8.0)

Procedure

- Prepare the mix at the following final concentration (total volume 25 µL):
 - 100-250 ng DNA
 - 200 nM dNTPs
 - 0.1 µM of each primer
 - 1.5 U *Taq* polymerase
 - PCR buffer (with MgCl₂), 8% (v/v)
 - sterile H₂O, 46.4% (v/v)
- Program the automated thermal cycler according to manufacturers' instructions. Use the following guidelines to set annealing temperature and extension time according to primer and product considerations.
- Hot start at 95 °C for 10 min when using *Taq* Gold.
- Denature the DNA for 1 min at 94 °C. If the GC content is ≤50%, anneal it at 55 °C; if it's >50%, anneal at 65 °C. Extend for 5 min at 72 °C. Run the program for 30 cycles.
- Prepare 2% agarose gel in TE buffer. Run the gel at 80 to 100 V and stain it with 0.1% ethidium bromide. Visualize the gel under UV light. Take a photograph.

VI:C. PCR for amplification of single nucleotide polymorphisms or RFLP of cytokine genes

by **Ben Gyan**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: bgyan@noguchi.mimcom.net

Procedure

- Use the same procedure as for VNTR polymorphisms, CELLULAR IMMUNOLOGY, section VI:B. The PCR product will give the same band for all samples. To detect polymorphisms, further digest the PCR product with digestive enzymes according to the manufacturers' instructions.
- Run the digested PCR product on 3% agarose gel (use NuSieve GTG) electrophoresis at 80 V and stain the gel with 0.1% ethidium bromide. Visualize the gell under UV light and take a photograph

VI:D. Amplification Refractory Mutation System (ARMS) PCR for detection of previously characterised mutations in cytokine genes

by **Ben Gyan**

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

e-mail: bgyan@noguchi.mimcom.net

Equipment

automated thermal cycler
agarose gel electrophoresis
UV-transilluminator

Materials and reagents

Tris-HCl (pH 8.8)

MgCl₂

Taq polymerase

dNTP

Tween 20 (ABgene)

primers

ethidium bromide

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

PCR master mix:

25 mM Tris-HCl (pH 8.8)

1.5 to 4.0 mM MgCl₂

0.25 *Taq* polymerase

200 µM dNTP

0.01% (v/v) Tween 20 (ABgene)

Primer mix:

specific primer mix (10 µM of generic primer and 10 µM of specific primer)

5 µM of positive internal control (10 µM of internal control forward and 10 µM of reverse primers).

Procedure

- Put 100 to 250 ng of each DNA into two tubes or wells.
- Add other PCR reagents as indicated in the master mix above.
- Program the automated thermal cycler according to manufacturers' instructions.
- Use guidelines in CELLULAR IMMUNOLOGY, section VI:B for VNTR polymorphisms to set annealing temperature and extension time according to primer and product considerations. Run the program for 30 cycles.
- Analyze the PCR products by agarose gel electrophoresis followed by ethidium bromide staining and visualisation under UV light.

Amplification should occur in only one tube if the DNA template contains wild-type sequence or mutation sequence. Heterozygous individuals show amplification in both tubes. A positive control sequence is required to measure that absence of product is not due to PCR failure.

Reference

Perrey C, Turner SJ, Pravica V, Howell WM, Hutchinson IV. 1999. ARMS-PCR methodologies to determine IL-10, TNF-alpha, TNF-beta and TGF-beta 1 gene polymorphisms. *Transpl Immunol* 7(2):127-128.

VII. Haptoglobin phenotyping using PAGE

by **Ben Gyan**

Immunology, Stockholm University, SE-106 91 Stockholm,

e-mail: bgyan@noguchi.mimcom.net

Equipment

gel electrophoresis apparatus
gel drier or oven

Materials and reagents

polyacrylamide gel
Tris-HCl
Tris-base
glycine
distilled water
10% ammonium persulphate
TEMED (tetramethyl-ethylenediamine)
haptoglobin
erythrocyte haemolysate
benzidine stain
filter paper

Buffers:

1.5 M Tris-HCl (pH 8.8)
54.5 g Tris-base
150 mL distilled H₂O

0.5 M Tris-HCl (pH 6.8)
6 g Tris-base
60 mL distilled H₂O

10× running buffer
15.5 g Tris-base
72.1 g glycine
500 mL distilled H₂O

Procedures and preparations

- Cast a polyacrylamide gel with separation gel of concentration 4.7% and stacking gel of 2.5%.

	<u>separating gel (4.7%)</u>	<u>stacking gel (2.5%)</u>
30% bis acrylamide	7 mL	1.3 mL
Tris-HCl	11.25 mL	2.5 mL
distilled water	26.7 mL	6.0 mL
10% ammonium persulphate	150 µL	50 µL
TEMED	37.5 µL	18 µL

- Add 3 µL of erythrocyte haemolysate to 10 µL of serum or plasma and control haptoglobin.

- Mix the solution well and incubate it for 5 min to enable haptoglobin in serum to bind to hemoglobin.
- Add 10 μ L of loading buffer. Load the sample onto the gel using a template.
- Run electrophoresis at a constant voltage of 80 to 120V using 1 $\times$ running buffer.
- Stain the gel for 5 to 15 min with benzidine stain.
- Wash the gel in distilled water.
- Transfer the gel onto filter paper and dry it using a gel dryer or oven.

References

Davis I, Orstein J. 1968. Disc electrophoresis, acrylamide gel columns: *In*: Williams, CA, Chase MW, eds. Methods in Immunology and Immunochemistry. Vol. 2. New York: Academic Press; pp. 34-47.

Coligan JE, et al., eds. 2003. Current protocols in immunology. Vol. 2. Electrophoretic separation of proteins. Chapter 8, section III. New York: Wiley-Interscience.

MOLECULAR BIOLOGY

I. Small-scale genomic DNA isolation from *Plasmodium falciparum*

by **Martha Schlichtherle** and **Mats Wahlgren**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm Sweden

e-mail: mats.wahlgren@mtc.ki.se

Equipment

centrifuge (4 °C)
speed-vac
agarose electrophoresis unit
spectrophotometer

Materials and reagents

infected erythrocytes (a few mL with about 10% parasitemia)
phosphate-buffered saline (PBS) (pH 7.2)
5% saponin solution
lysis buffer:
 40 mM Tris-HCl (pH 8.0)
 80 mM EDTA (pH 8.0)
 2% SDS, 0.1 mg/mL
proteinase K (Add the proteinase K just before using the buffer.)
phenol equilibrated with 0.1 M Tris-HCl (pH 7.0)
chloroform
RNase (Stratagene, RNase-It Ribonuclease Cocktail)
3 M sodium acetate (pH 5.0)
absolute ethanol
70% ethanol
TE-buffer:
 10 mM Tris-HCl (pH 8.0)
 1 mM EDTA (pH 8.0)

Procedure

- Centrifuge the infected erythrocytes at $3,000 \times g$ for 2 min. Wash the cells once in cold PBS.
- Resuspend the cells from one microfuge tube (1.7 mL) in 1 mL of PBS.
- Add and gently mix 10 μ L of 5% saponin (for a final concentration of 0.05%).
- Immediately centrifuge the tube at $6,000 \times g$ for 5 min after lysis is observed.
- Remove the supernatant.
- Add 25 μ L of lysis buffer and 75 μ L of distilled water to the pellet.
- Incubate the tube at 37 °C for ~3 h with intermittent stirring by hand.
- Add 100 μ L of distilled water, then 200 μ L of phenol. Mix well and centrifuge at $2,000 \times g$ for 8 min.
- Extract likewise with 200 μ L of chloroform.
- Add 2 μ L of RNase-It cocktail for 30 min at 37 °C.
- Extract with phenol and chloroform as above.

- Precipitate the gDNA by adding one-tenth volume of sodium acetate and 2.5 volumes of absolute ethanol; allow the tube to sit for 2 hours or overnight at -20°C . The DNA can be stored this way as well.
- Centrifuge the precipitate at $2,000 \times g$ for 30 min at 4°C , wash it gently once with 70% ethanol, dry it in a speed-vac, and gently resuspend the pellet in 25 to 100 μL of distilled water, depending on its size.
- Determine the DNA concentration at OD_{260} ; 2 mL of culture may yield $\sim 2 \mu\text{g}$ of gDNA. Visualize $\sim 0.5 \mu\text{g}$ on a 0.8% agarose gel to see that the gDNA runs as a high-molecular weight, somewhat broad band and is thus unsheared and free of RNA.

II. RNA extraction from *Plasmodium falciparum*

II:A. RNA extraction from *Plasmodium falciparum* using guanidine thiocyanate and acidic phenol

by **Bobo Mok** and **Martha Schlichtherle**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: bobo.mok@mtc.ki.se

Equipment

centrifuge (4 °C)
JA-20 Beckman rotor
small-blade Polytron homogenizer (Brinkmann)

Materials and reagents (see also Solutions below)

PRBC (Check PRBC to determine the parasitemia and rosetting rate. Usually eight 75-cm² bottles with a parasitemia of 7 to 10% will yield ~200 to 300 µg of RNA.)
cold sterile PBS (pH 7.2)
complete solution D (with 72 µL 2-mercaptoethanol (2-ME)/10 mL of Solution D or 288 µL of 2-ME/40 mL of Solution D). Remove 1 mL into a microcentrifuge tube; store the rest at 4 °C for Day 2. Solution D with 2-ME added is stable for 2 weeks at 4 °C.)
2 M sterile sodium acetate (pH 4)
acidic phenol:chloroform:isoamylalcohol (125:24:1) (Ambion 9722)
ice cold 2-propanol
distilled water
50-mL Falcon tubes (BD Labware)
tubes (see under MOLECULAR BIOLOGY, sections II:C)

Solutions

Solution D for RNA isolation:

100 g guanidine thiocyanate
117.2 mL sterile distilled water
7 mL 0.75 M sodium citrate (pH 7.0) (autoclaved)
10.6 mL 10% N-lauroyl-sarcosine (Sigma L9150; filtered; store stock at room temperature)
Check the pH, which should be ~7.0.
The solution can be stored for 3 months at room temperature.

Working solution:

720 µL 2-mercaptoethanol/100 mL of Solution D; keeps for 2 weeks if stored at 4 °C.

2 M sodium acetate, 50 mL:

Add 8.2 g of anhydrous sodium acetate to about half the final volume of distilled water; as the salt is dissolving, adjust the pH to 4.0 with ~16 mL of concentrated acetic acid; bring to volume and autoclave.

Tris–EDTA buffer (TE) (see also Sambrook et al. 1989):

10 mL 1 M Tris–HCl

2 mL 0.5 M EDTA (pH 8.0)

988 mL water

Combine Tris and EDTA in water; adjust pH to 7.4; autoclave. Store the solution at 4 °C; it is stable for 6 months.

10× electrophoresis buffer (0.2 M MOPS and 10 mM EDTA):

40 mL 1 M MOPS (pH 7.4) (autoclaved; 209.3 g/mol)

4 mL 0.5 M EDTA (pH 8.0) (autoclaved)

156 mL distilled water (autoclaved)

RNA loading buffer, deionized formamide:

25 g mixed-bead ion exchange resin (Bio-Rad, 142-6424)

250 mL of formamide (Sigma F7503)

Wrap an Erlenmeyer flask in foil. Add resin beads and formamide. Stir the mixture for 60 min in the hood. Filter through Whatman No. 1 filter paper. Store aliquots in dark bottles at –20 °C (stable for 1 year).

RNA loading buffer:

3.37 mL deionized formamide

1.08 mL formaldehyde (37%)

0.5 mL 10× electrophoresis buffer

20 µL 0.5 M EDTA (pH 8.0)

285 µL glycerol (sterile)

50 µL 10% SDS

50 µL bromphenol blue, 0.5% (or 5 mg)

50 µL xylene cyanole, 0.5% (or 5 mg)

Aliquot, then store at –20 °C.

Agarose gel (small 40 mL):

0.4 g agarose (Sigma A9539)

29 mL distilled water

Add agarose to sterile water, heat to boiling to dissolve the agarose, then cool to 65 °C.

Add:

4 mL 10× electrophoresis buffer

50 µL 0.5 M EDTA

In the hood, quickly add 7 mL of formaldehyde (or less if using Sigma's buffers).

Pour the gel solution into a clean, taped gel tray containing a comb. Remove bubbles and allow the gel to cool.

20× SSC stock (5 L):

876.5 g sodium chloride

441.0 g sodium citrate

Add salts to distilled water and mix to dissolve. Add distilled water to total of 5 liters, adjust the pH to 7.0 with HCl, and autoclave.

Prehybridization/hybridization fluid:

0.5 M Na_2HPO_4 (pH 7)
1 mM EDTA
7% SDS

Probe removal solution

0.1% SDS

Place a nylon filter in the boiling solution and remove from heat. After 15 min remove and check the filter. Either repeat the treatment or simply expose the filter to check that the probe is really removed. Keep the filter moist at all times by placing it in a sealed hybridization bag. It may be stored at -20°C .

Isolation of RNA, Day 1

- Cool the centrifuge for 50-mL Falcon tubes.
- Pool cells (PRBC) into two 50-mL tubes on ice.
- Centrifuge the cells at 1,800 rpm for 5 min at 4°C .
- Remove the supernatant. Wash the cells with ~ 30 mL of cold PBS. Gently resuspend the cells. Centrifuge them again.
- Remove the supernatant. Resuspend the cells in cold PBS to ~ 20 mL/tube and transfer into centrifuge tubes that have lids (i.e., not Corex tubes) and that withstand $15,000 \times g$ and organic solvents. Centrifuge them as above.
- Remove the supernatant and set the cells on ice.
- Rinse and clean a small-blade Polytron homogenizer by running distilled water in it, followed by some solution D.
- Add 10 mL of solution D per centrifuge tube and suck up and down to disrupt cells.
- Homogenize the cells on “medium” setting for 15 s. Avoid frothing. Keep the cells on ice.
- Clean the homogenizer by running solution D in it, then running distilled water, with changes of water until no trace of homogenized material appears on the blades.
- To each tube add:
 - 1.0 mL 2 M sodium acetate (pH 4) ($\times 0.1$ volume)
 - 10 mL acidic phenol ($\times 1$ volume)
 - 2.0 mL chloroform:isoamyl alcohol ($\times 0.2$ volume)
- Cap the tubes with lids and vortex well for ~ 1 min. Set them on ice for 15 to 30 min. Meanwhile cool the centrifuge and rotor (JA-20 Beckman rotor) to 4°C .
- Invert and vortex to mix the samples, then immediately centrifuge the tubes at 11,000 rpm for 30 min at 4°C .
- Remove and save the supernatant in two new centrifuge tubes (Nalgene 3110). Use lids. Avoid material from the interphase.
- Add an equal volume of 2-propanol (at -20°C), mix, and set the sample at -20°C overnight or at least for a few hours.

Further purification of RNA, Day 2

- Cool the JA-20 Beckman rotor. Mark the side of each tube where the “invisible” RNA pellet is expected to be. Centrifuge the tubes at 11,000 rpm for 30 min at 4 °C.
- In a ventilated hood, carefully remove the supernatant with a 10-mL pipette, being careful to go down the opposite side of the tube from where the pellet is expected. There should be an almost transparent, gel-like pellet. Mark the tube where the pellet is.
- Dry the tubes some in the hood, but do not over-dry them.
- Resuspend both tubes’ RNA contents in a total of 0.5 mL of Solution D.
- Transfer the contents into a microcentrifuge tube. Precipitate it with an equal amount of 2-propanol overnight at –20 °C (or at least for a few hours).

References

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 162(1):156-159.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

II:B. Measurement of RNA yield and purity, and visualization on agarose–formaldehyde gel

by **Bobo Mok** and **Martha Schlichtherle**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm Sweden

e-mail: bobo.mok@mtc.ki.se

Equipment

microfuge

Materials and reagents

premixed RNA electrophoresis buffer (Sigma M5755)

RNA gel loading buffer (Sigma R4268)

10× electrophoresis buffer:

23.4 mL distilled water

600 µL 0.5 M EDTA (pH 8)

6 mL 1 M MOPS (pH 7.4)

This makes 30 mL total, approximately the amount needed for a regular small gel system.

formaldehyde

agarose

1 M EDTA (pH 8.0)

distilled water (or DEPC-treated water)

3 M sodium acetate (pH 5.2)

ethidium bromide

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

bromphenol dye

Procedure

- Extract and purify the RNA as instructed in MOLECULAR BIOLOGY, section II:A, Days 1 and 2.

Day 3:

- Microfuge the RNA for 30 min at 12,000 to 14,000 rpm and 4 °C.
- Meanwhile prepare buffers and gel. Use EITHER:
 - premixed RNA electrophoresis buffer (Sigma M5755) and RNA gel loading buffer (Sigma R4268). The gel should then contain a lower amount of formaldehyde of 2.4%.
 - OR:
 - 10× electrophoresis buffer
- Have formaldehyde ready in the hood.
- Prepare a 1% agarose gel:
 - 0.4 g agarose
 - 29 mL of distilled waterMix, heat, and cook briefly. Cool gel liquid some. Then add:
 - 4 mL 10× electrophoresis buffer
 - 25 µL 1 M EDTA (pH 8.0)

- Bring the gel liquid to the hood, add 7 mL of formaldehyde, mix, then quickly pour the gel. Use a comb which is set aside for RNA-work so that no RNases from fingers are present on it.
- Allow the gel to solidify in the hood, but do not over-dry.
- Continue with the RNA sample. Wash precipitated RNA 2 times with 900 μ L of 70% ethanol at -20°C . Allow it to dry some in the hood.
- Set a small water bath to 60 to 65°C .
- Dissolve the RNA in ~ 100 μ L of autoclaved distilled water (or DEPC-treated water) depending on pellet size. If the RNA does not dissolve well with pipetting, heat it briefly at 65°C . Pipette to dissolve, then keep the RNA on ice.
- Use $\sim 1:100$ dilution of RNA for spectrophotometric measurement, preferably in a microcuvette to minimize the amount of RNA which is lost to absorbance measurements.
- Use the program for fixed wavelength at 260 and 280 nm.
- Take absorbance readings after blanking with distilled water.
- Calculate:

$$\text{RNA concentration of stock: (Abs) (dil.) (40 } \mu\text{g/mL)}/1000 = \text{RNA } \mu\text{g}/\mu\text{L}$$

$$\text{total yield: (above \#) (}\mu\text{L stock RNA) = } \mu\text{g RNA}$$

$$\text{purity: Abs}_{260}/\text{Abs}_{280} = \text{should be } \sim 1.8 \text{ to } 2.0 \text{ if not too much protein contamination is present.}$$
- Take out 5 μ g or less of RNA to run in a lane on the gel.
- The RNA left to precipitate is: total yield – spec. reading – gel loading.
- Precipitate the RNA left by adding one-tenth volume of 3 M sodium acetate (pH 5.2), mix, then add 3 times volume of -20°C absolute ethanol. Store RNA at -70°C .
- If RNA volume taken out for gel is >10 μ L, speed-vac it down to dryness. Otherwise add RNA loading buffer to 19 μ L (or less if using Sigma's premade solution, see manufacturer's suggestions). Mix and heat for 5 to 10 min at 60 to 65°C . Set on ice
- Add 1 μ L of ethidium bromide (at ~ 250 ng/ml); mix. Pour $1\times$ electrophoresis running buffer over the gel and load.
- Run the gel at ~ 50 V for more than 1 h with the bromphenol dye running about half way. Use commercial RNA marker(s) if needed (i.e., Gibco). For best results the gel should be run at 4°C . View and photograph the gel under UV-light. Two RNA bands should be quite visible corresponding to rRNAs. A third low rRNA band may at times also be visible; it is usually fuzzy. After a distilled-water rinse, the gel may be transferred to a nylon filter and be Northern probed using manufacturer's protocols.

References

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 162(1):156-159.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

II:C. Reliable RNA preparation for *Plasmodium falciparum*by **Sue Kyes**Molecular Parasitology Group, Weatherall Institute of Molecular Medicine, Headington,
Oxford OX3 9DS, UKe-mail: skyes@molbiol.ox.ac.ukEquipment

benchtop centrifuge, refrigerated
 microfuge, ambient or refrigerated
 water baths/heating blocks (37 °C and 60 °C)
 spectrophotometer/cuvettes for reading absorbance at 260 nm and 280 nm
 (optional)

Materials and reagents

parasite-infected red blood cells (iRBC):

0.5 mL packed cell volume at 10% parasitemia, expected yields:

rings 50–150 µg total RNA

trophozoites, schizonts, gametocytes 250–750 µg total RNA

gloves

TRIzol Reagent: (Invitrogen) 15596-026 (Follow manufacturer's suggestions for
safety and storage.)

chloroform (AnalAR from BDH, or Sigma C5312, or similar)

2-propanol (AnalAR from BDH, or similar)

formamide (molecular biology grade, Sigma F9037, or similar. It is not necessary
to deionize before use. Open bottle in chemical fume hood.)

RNase-free, chloroform/phenol-resistant plastics

screw-top test tubes*

1.5-mL microfuge tubes*

pipette tips*

**Straight from manufacturer is usually ok; autoclave if not sterile, taking care
to wear gloves in handling.*

optional:

RNase-free water (Sigma W4502 or Milli-Q purified or similar)

RNase-free 3M sodium acetate solution (pH 5.2) (Sigma S7899 or similar)

Either buy RNase-free water and sodium acetate solution or treat
distilled water and sodium acetate solution with 0.1% diethyl
pyrocarbonate (DEPC; Sigma, D5758) as described in Sambrook et al.
1989; p. 7.3; this requires autoclaving.

RNase-free 75% ethanol/25% RNase-free water

RNase-free absolute ethanol (AnalAR from BDH, or Sigma E7023, or
similar)Protocol**Key RNA advice: Wear gloves for everything, and use RNase-free plastics.**Preserve RNA in TRIzol

- Spin cells gently at 1,800 rpm ($600 \times g$) for 4 min at room temperature.
(example: 0.5 mL of packed infected RBC, about 10% parasitemia)^{1,2}
- Remove supernatant (s/n)³. Tap the tube to loosen the cell pellet⁴.

- Add TRIzol, prewarmed to 37 °C⁵.
For RINGS: add 10 pellet volumes of TRIzol.
(*example: 5 mL of TRIzol to 0.5 mL cells*)
For all other stages: add 20 pellet volumes of TRIzol.
(*example: 10 mL of TRIzol to 0.5 mL cells*)
- Shake the sample to dissolve any clumps.
- Incubate the sample at 37 °C for 5 min.
- Either store the sample at –70 to –80 °C⁶ or continue.

Continue extraction and precipitate RNA

- (Thaw samples at 37 °C, if necessary.)
- Add 0.2 TRIzol volumes of chloroform.
(*example: sample in 5 mL TRIzol, add 1 mL of chloroform*)
(*example: sample in 10 mL TRIzol, add 2 mL of chloroform*)
- Vigorously shake the sample; let it stand at room temperature for 2 to 3 min.
- Spin the tube at 4 °C for 30 min (microfuge = maximum 9,000 × *g*; or for 15-mL tubes in benchtop centrifuge, 1,000 to 1,400 × *g* is fine)⁷.
- Remove the aqueous (top, clear, RNA-containing) layer to a new tube⁸. Expect to recover up to 0.6 TRIzol volumes, but avoid the interface!
(*example: from sample in 5 mL of TRIzol, expect 3 mL of aqueous layer*)
- Add 0.5 TRIzol volumes of 2-propanol to the RNA; invert the tube several times to mix.
(*example: to 3 mL of aqueous layer, add 2.5 mL 2-propanol*)
- Transfer/split large samples to 1.5-mL snap-cap microfuge tubes.
- Precipitate the samples for AT LEAST 2 h (up to several days) at 4 °C.

EXAMPLE
Beginning with
0.5 mL of iRBC,

to the rings (*trophs*)
↓
add TRIzol
5 mL (10 mL)
↓
and chloroform
1 mL (2 mL).

Sample size is now:
3 mL (6 mL).

Add 2-propanol
2.5 mL (5 mL).

Finish preparation, check quantity/ quality of RNA

- Spin the sample at 12,000 × *g* (13,000 to 14,000 rpm, microfuge) for 30 min at 4 °C or room temperature⁹.
- Remove as much as possible of the supernatant¹⁰ (*Optional 75% ethanol wash¹¹).
- Air dry the sample, inverted, at room temperature, for no more than 5 min.
- Add formamide (usually a total of 100 µL for a starting pellet of 0.5 mL of infected RBC)¹².
- Heat the sample at 60 °C for 10 min; place it on ice.
- Resuspend the pellet by pipetting¹³.
- Check the absorbance at 260 nm¹⁴.
- Store the pellet at –70 to –80 °C.

Notes to the above procedure:

- 1: Especially for rings, cells pellet much more efficiently in 15-mL tubes than in 50-mL tubes. Subsequent steps are also easiest in 15-mL tubes, so harvest large volumes of cells directly in 15-mL tubes.
- 2: Use phenol/chloroform-resistant tubes.

- 3: After removing tubes, adjust centrifuge temperature down to 4 °C for the next spin.
- 4: Do not wash cells or lyse them with saponin. It is important to lyse the infected red blood cells directly in TRIzol as soon as possible after removing them from culture.
- 5: TRIzol seems to work best if it is prewarmed to 37 °C. Make sure lids are tightly sealed before shaking the tubes. Do the 37 °C incubation *before* freezing the samples to ensure complete complexing of nucleoproteins. The TRIzol can cope with extracting the RNA from hemoglobin, of which there is obviously plenty. TRIzol does not cope with separating RNA from DNA very well. Trophozoites/late stage parasites synthesize lots of DNA, and therefore require excess TRIzol.
- 6: Because the RNA is very stable at this point (if stored at –70 to –80 °C), collecting lots of samples is easy. Store them until you can process them all at once.
- 7: Do NOT spin at room temperature. If using a microfuge, use screw-cap tubes. Snap-cap tubes tend to leak and make a horrible mess of the inside of the centrifuge.
- 8: Avoid the interface to avoid DNA contamination. It is easier to recover more of the aqueous layer from 15-mL tubes than from 50-mL tubes. The DNA seems to collect right *above* the interface, so do not try to collect every last microliter of aqueous layer. The manufacturer's protocol for retrieving DNA from this does not seem to work due to massive amounts of hemoglobin. If anyone manages, please let me know!
- 9: Give tubes a quick vortex before spinning. Place tubes in the microfuge with hinges pointing outwards (for future identification of RNA pellet). Room temperature spin is fine, but 4 °C is ideal.
- 10: Remove supernatant in two stages:
 - a. Keeping pipette tip away from the putative pellet (directly beneath hinge, at bottom of tube), remove nearly all of the supernatant. Save the supernatant to a new tube if you are worried about losing your sample.
 - b. Give the tube a quick 'flick spin', again with hinges pointing outwards. This gets all those dribbles on the side of the tube down to the bottom again. Remove last bits of supernatant, as much as possible, taking care to avoid touching the very tiny, glassy pellet. If there is a large pellet, there is probably DNA or protein in it.
- 11: At this point, it is optional to add a 75% ethanol wash step: Add 0.5 mL of 75% ethanol: 25% DEPC–water, ice-cold; spin for ~5 min in a microfuge, and remove all supernatant. This gets rid of all traces of phenol and is particularly useful for cDNA preparations (see next note).
- 12: Add the formamide to the tube, avoiding touching the 'pellet'. Split the total formamide volume among the microfuge tubes if it was a large sample split to several tubes. RNA is very stable for a long time in formamide. However, the formamide affects downstream enzymatic applications. If you plan to make cDNA, it is worth considering resuspending the RNA pellet in DEPC–water, if you have it. If you have RNA samples in DEPC–water, store them in aliquots at –80 °C, but beware that the RNA is not very stable in water, and does not survive many freeze-thaw cycles. One way around this is to precipitate the RNA (add one-tenth volume of RNase-free 3M sodium acetate [pH 5.2], and 2.5 volumes of ethanol), then store at –80 °C. This is very stable, and will last 'forever'. When

- you need some RNA, just thaw the tube, vortex it, and remove the volume necessary to give the desired amount; spin it down in the microfuge for ~30 min.
- 13: By pipetting up and down, mix each sample thoroughly, making sure that nothing is stuck to the bottom of the tube. The pellet should have completely dissolved. Pool tubes of the same sample-type back together at this point. Set aside small volumes for measuring absorbance or running on gel.
- 14: Spectrophotometric measurement of quantity:
Check absorbance at 260 nm: Try 1 μ L in 500 μ L of water, remembering to blank against 1 μ L of formamide in 0.5 mL of water.
Conversion: OD 1 = 40 μ g RNA
Compare to OD 280. The ratio OD260/OD280 should be close to 2.0 for pure RNA. If there is any contaminating phenol, this number will be lower and the quantification will not be accurate.
If this of great concern, at the point marked *above, add the 75% ethanol wash step. It does not seem to make much difference, but gives some people peace of mind that the RNA is really clean.

Agarose gel measure of quality:

Using the procedure outlined for Northern blots below (see MOLECULAR BIOLOGY, section II:D), run a small gel, with small lanes. Run 1 to 2 μ L of each sample.

There should be two main ribosomal bands and a few minor bands of unknown provenance. Good RNA preparations have roughly equal amounts of both main ribosomal bands. If the RNA is degraded, there will be a 'comet tail' and no discrete bands. If there is plenty of DNA, there will also be a faint smudge close to the wells. Sometimes this does not matter, but sometimes it is really annoying. There are methods for getting rid of the DNA, but they are expensive and not fun.

Finally, if your sample had human white blood cells in it, you'll see more than two ribosomal bands!

References

Kyes S, Pinches R, Newbold C. 2000. A simple RNA analysis method shows *var* and *rif* multigene family expression patterns in *Plasmodium falciparum*. Mol Biochem Parasitol 105(2):311-315.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 7.3-7.4.

TRIzol is based on the following:

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162(1):156-159.

II:D. Simple Northernsby **Sue Kyes***Molecular Parasitology Group, Weatherall Institute of Molecular Medicine, Headington,
Oxford OX3 9DS, UK**e-mail: skyes@molbiol.ox.ac.uk***NO DEPC-treatment anywhere! Just wear gloves for everything.**Equipment

agarose gel tank, combs, and power pack
deep plastic trays for soaking gels (rinsed with 1× distilled water)
UV transilluminator and gel photography equipment
heating block/water bath (60 to 65 °C)
ice to chill samples

Materials and reagents

gloves
hydrogen peroxide, 30% (Sigma H1009)
deionized water (or 1× or 2× distilled water), but not autoclaved or DEPC-treated,
for all gel buffers and solutions, except as noted*.
No solutions are autoclaved

1× TBE (as for DNA gels):

0.089 M Tris
0.089 M boric acid
2 mM EDTA

5× TBE:

54 g Tris-base
27.5 g boric acid
20 mL 0.5 M EDTA
Combine ingredients; makes 1 liter.

agarose (normal grade as for DNA gels)
1 M guanidine thiocyanate (Sigma G6639; MW=118.2); i.e., 118 mg/mL of
sterile* water (non-DEPC, as used for DNA work is fine)
Make only as much as you need on day of use (e.g., ~0.5 to 0.6 mL).
TOXIC; be sure to dispose of waste properly.

DNA loading dye containing bromophenol blue
formamide (Sigma F9037, molecular biology grade).
Not necessary to deionize. Open in chemical fume hood.

fresh, dilute ethidium bromide solution (~0.1 to 0.5 µg /mL, ~200 mL)
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and
use only in a chemical fume hood.

fresh 7.5 mM NaOH, ~1 to 1.5 liter (fresh, made on same day)
for blotting (see Sambrook et al.)
Hybond N+ (Amersham)

Whatman 3M paper
paper towels
2 glass plates, one big enough to fit over a shallow plastic tray
shallow plastic tray
2× SSC:
0.3 M NaCl
0.03 M sodium citrate
(1 liter of 20× SSC = 175.3 g NaCl, 88.2 g sodium citrate, to pH 7 with HCl; dilute 1:10 for 2× SSC)

Protocol

Electrophoresis of RNA and capillary transfer to nylon membrane

- Clean gel apparatus by soaking everything including combs in 3% H₂O₂ for 10 min (1:10 dilution of 30% stock in 1× distilled H₂O)
or in 0.5 M NaOH for 2 h,
or in 1% SDS for 2 h
then rinsing in 1× distilled H₂O (which is not necessarily sterile or RNase-free!).
- For a 100-mL, 1% agarose gel, using a relatively clean bottle, combine 100 mL of 1× TBE + 1 g agarose, and heat, with caution, in a microwave oven .
The best resolution depends on the expected transcript size. Use the following guide for agarose concentration:
0.8% agarose for large transcripts (greater than 7 kb)
2% agarose for short transcripts (1.5 to 3 kb)
- Cool the gel to 55 to 60 °C. Check this with a relatively clean thermometer.
- Add 0.5 mL of freshly-made 1 M guanidine thiocyanate (final concentration = 5 mM G-SCN).
- Pour the gel and let it set for at least 30 min at room temperature.
- Prepare the samples while the gel sets.
amount of RNA:
For 0.8% agarose gel, use 6 to 10 µg of total RNA per lane.
For 2% agarose gel, use 3 to 5 µg per lane.
volume of RNA to load:
total, approximately 5 to 20 µL per lane
loading dye:
Not necessary if samples are in formamide.
If your samples are in water, add an equal volume of formamide (final 50% formamide or greater).
NOTE: Don't put formaldehyde in your samples. It does not work on this type of gel!
If you absolutely need loading dye, try formamide with final 1× TBE, 0.5% bromophenol blue. Add this AFTER denaturing your RNA in formamide.
- Denature ~2 µg of size-standard RNA, in ~10 µL of formamide for ~10 min; place it on ice.
- Denature samples at 60 to 65 °C for 2 min; place it on ice.
- Having rinsed the gel tank, place the set gel in it, pour in 1× TBE to cover it, then remove the comb and any end blocks.
- Load the gel. Load a blank lane with DNA loading dye between the size marker and the samples to track the running of the samples. This also keeps your

marker from spilling into a sample lane, which sometimes makes a mess of hybridizations.

- Electrophorese samples into the gel for approximately 10 to 15 min at 110 volts, then run at 70 to 80 volts (3.5 v/cm) until the bromophenol blue is approximately 8 to 10 cm from the well. The process takes about 3 to 4 h.
- Stain the samples in fresh, dilute, ethidium bromide solution for ~10 min.
- Destain the for a few minutes in 1× TBE using buffer left over from the gel box.
- Photograph them with a ruler next to the gel. Leave the gel on UV transilluminator for ~2 min. This nicks the RNA, helping with transfer of larger transcripts.
- Soak the gel in ~200 mL of 7.5 mM NaOH, twice, for 10 min each time.
- Set up the capillary transfer to Hybond N+ in 7.5 mM NaOH to run overnight (not less than 8 h, especially important for large transcripts). (See Sambrook et al. 1989.)

Disassemble blot and start hybridization

- Neutralize the filter in 2× SSC for 5 min.
- Air dry the filter. Put the filter on UV light box at this point and draw size marker positions directly on the filter with a pencil.
- UV-crosslink, if you have a cross-linker, but this is not necessary.
- Prehybridize and hybridize as usual (see Kyes et al. 2000 for solutions and conditions). To start, try 55 °C hybridization, 60 °C wash in 0.5× SSC/0.1% SDS.

References

Goda S, Minton N. 1995. A simple procedure for gel electrophoresis and northern blotting of RNA. *Nucleic Acids Res* 23(16):3357-3358.

Kyes S, Pinches R, Newbold C. 2000. A simple RNA analysis method shows *var* and *rif* multigene family expression patterns in *Plasmodium falciparum*. *Mol Biochem Parasitol* 105(2):311-315.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; pp. 7.46-7.49.

III. *Plasmodium falciparum* cDNA library construction

by **Martha Schlichtherle** and **Mats Wahlgren**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: mats.wahlgren@mtc.ki.se

Equipment

centrifuge (4 °C)
agarose electrophoresis unit
50-mL Falcon tubes (BD Labware)
Polytron homogenizer (Brinkmann)
centrifuge (Beckman)
speed-vac

Materials and reagents

Consider using kits from Stratagene for extraction of RNA and construction of cDNA libraries. They are generally more commonly employed than the Pharmacia kits used below. However, we did successfully make several cDNA libraries from two different strains using the Pharmacia kits.

Throughout the following protocols (MOLECULAR BIOLOGY III:A–E), there are references to the appropriate protocols from the companies from which library materials were bought. Further changes in these general “company-protocols” have been outlined below to facilitate *Plasmodium falciparum* library construction.

III:A. Cell preparations at desired cell stage(s)

Grow parasites in 75-cm² large flasks (12 mL per flask). You will need several sets of large flasks to be able to purify a workable amount of mRNA. We usually used 8 bottles/preparation where 8 bottles with ~10% parasitemia would yield 100 to 200 µg (sometimes even 300 µg) of total RNA. If rosetting parasites are desired, use parasites with $\geq 50\%$ rosetting rate. See also protocols on cell culturing in PARASITES, section I, and synchronization of erythrocytic stages if that is desired (PARASITES, section IV).

III:B. RNA preparation (total RNA)

See separate protocol for RNA preparation in MOLECULAR BIOLOGY, section II above. Store RNA as a precipitate in 3× volume of ethanol with 0.3 M sodium acetate at –70 °C in microfuge tubes.

III:C. mRNA selection of pooled total RNA

See general protocol from 5Prime → 3Prime for mRNA isolation using oligo(dT) cellulose spin columns. (See also other companies for smaller amounts of mRNA isolation.)

Procedure

- Centrifuge microfuge tubes with RNA in microfuge at 4 °C for 30 min.
- Wash samples with 70% cold ethanol and centrifuge them again for ~20 min.
- Dry tubes upside down in a hood with good airflow or in a speed-vac.

- Add 50 to 100 μL of pure “kit- H_2O ” to each tube and resuspend the RNA. Pool the RNA into one tube.
- Heat the sample at 65 $^{\circ}\text{C}$ for a few minutes, then place it on ice.
- Load 1 to 5 mg of total RNA per column. See company protocol (5Prime $\rightarrow$ 3Prime) for the rest.

Examples of mRNA yields:

One sample of ~ 2 mg total RNA gave:

~ 87 μg mRNA after the 1st selection ($\sim 3.5\%$)

~ 61 μg mRNA after the 2nd selection ($\sim 2.5\%$)

Another sample of ~ 1.05 mg total RNA gave:

~ 71 μg mRNA after the 1st selection ($\sim 6.8\%$)

~ 42 μg mRNA after the 2nd selection ($\sim 4\%$)

From gel analysis it was clear that a second mRNA selection is not necessary since no rRNA bands were shown after one round of selection.

mRNA was stored as a precipitate according to the protocol with muscle glycogen, sodium acetate, and ethanol.

Store pure mRNA in aliquots of 1 μg , 5 μg , and maybe more.

Run a few microgram-per-lane samples to visualize mRNA as a faint smear in the range of the two larger rRNA bands. The front of these two bands should however be sharp if unselected total RNA is also run in a separate lane along with mRNA to indicate lack of degradation. See MOLECULAR BIOLOGY, section II:C for RNA-gel protocol. If using pure mRNA for a gel, you may want to blot and fix it to nylon for possible later use in a Northern.

III:D. cDNA synthesis

by **Martha Schlichtherle** and **Mats Wahlgren**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: mats.wahlgren@mtc.ki.se

Pharmacia's kits: Timesaver cDNA Synthesis Kit was used with some components replaced by the Directional Cloning Toolbox.

Additional reagents needed are indicated in the protocol booklet. If one has good quality distilled water, there is no need to use DEPC which is very hazardous (and can be destructive to RNA if it remains after autoclaving DEPC-treated water).

One needs to have access to the following water bath temperatures:

12 °C overnight (next day at 16 °C)

room temperature

37 °C

65 °C

Optional: dry ice for dry ice/ethanol bath.

Procedure

- Cool the lyophilizer trap and have spin columns ready in autoclaved, sterile Corex tubes.
- Use 4 µg of very pure mRNA per cDNA synthesis (both for FCR3S1 and TM284 strains). One could use 5 µg to possibly increase the recombinant phage.
- Centrifuge the mRNA in an Eppendorf microfuge for 30 min at 4 °C, then wash it gently once with 70% ethanol to remove salts from the small white mRNA pellet. Centrifuge it again for 20 min, then dry it briefly.
- Follow Pharmacia's general protocol; use 1 mL of *NotI* dT₁₈ primer from the Directional Cloning Toolbox as the cDNA primer. (See the Pharmacia protocol.)
- Where phenol/chloroform extraction is indicated, use 50 µL of each, followed by an extra step of pure chloroform extraction.
- Ligate the adaptor for 2 to 2.5 h at 16 °C.
- Digest the adaptor with *NotI* for 1.5 h (not 1 h), then extract it again with chloroform/phenol and pure chloroform.
- Precipitate the cDNA in an ethanol/dry ice bath for 30 min.
- If one uses two purification steps with organics (i.e., phenol/chloroform, then chloroform), as opposed to one combined step of phenol/chloroform, one loses more cDNA material.
- It may be appropriate to use 20 µL of column effluent with 2 µg of vector to do the ligations, rather than the 15-10-5 µL scheme suggested in the protocol, since 15 µL of effluent gave more recombinant phage than 10 µL, which gave more than 5 µL. One strain did not yield any recombinant phage with 5 µL of column eluate.

III:E. cDNA insertion, propagation, and amplification in phage using Pharmacia's λ ExCell NotI/EcoRI /CIP kit

by **Martha Schlichtherle** and **Mats Wahlgren**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: mats.wahlgren@mtc.ki.se

See Pharmacia's Timesaver cDNA Synthesis Kit protocol along with comments for cDNA and phage ligations. Use the phage λ vector to set up and do ligation reactions with *Plasmodium falciparum* cDNA.

Use extra microfuge spin times (20 min, not 10 min), 70% ethanol washing, centrifugation, and drying of DNA. cDNA/phage may be heated for a few minutes at 50 °C to sufficiently resuspend the cDNA before ligations are done. Incubate ligation reactions for 30 to 40 min at 16 °C, then at 4 °C overnight.

Use Stratagene's Gigapack II packaging extracts to package ligated products into phage. (Now there is the Gigapack III kit, which is easier to use).

Use 3 to 4 μ L of the cDNA- λ ligation per packaging. See the Gigapack II protocols.

Control packagings are not done. It is imperative to work very quickly once the extracts are removed from -70 °C to thaw. Package for 1 h 45 min.

Procedure

- Use *Escherichia coli* strain NM522 as the host for phage grown on special minimal plates (see Pharmacia protocols). Inoculate a few colonies in 10 to 20 mL of Luria broth (LB) with 0.2% maltose and 10 mM MgSO₄.
- Shake the inoculum at 30 °C overnight.
- The next day, centrifuge the cell suspension at ~2,000 rpm for 8 min and remove the LB medium.
- Resuspend the cells in 10 mM MgSO₄ for an OD₆₀₀ of 0.5.
- Cool the cells at 4 °C until use; use within 5 days with phage.
- Restreak stock plates of NM522 about every 3 weeks. Freeze extra bacterial stock at -70 °C as described in the manual using ~20% glycerol in the medium.
- Titer these primary libraries, 3 primary libraries/strain since there were 3 ligation mixtures set up for each strain. Phage dilutions of 1:10 and 1:100 of the primary libraries are recommended to titer.
- For the 1:10 dilution titering, mix 1 μ L of phage with 9 μ L of SM-buffer (see Stratagene's picoBlue Immunoscreening kit protocol).
- Make another such serial 1:10 dilution to achieve 1:100 phage dilution.
- Of each dilution, mix 1 μ L with 200 μ L of NM522 bacteria in 10 mM of MgSO₄ (see above) and place at 37 °C for ~15 min.
- Add 3 mL of LB low-melt agarose (at 49 °C) to the mixture and pour it on a prewarmed LB plate.
- Incubate the plate at 37 °C overnight.
- About 100 to 250 phage may be visible on the lower dilution plates. Approximately 0.5×10^6 to 1×10^6 independent clones may be generated per "ligation-library".

Amplification of primary libraries

- One needs about 40 to 50 large petri dishes with LB agar (poured fresh 1 to 2 days previously and evenly on a FLAT surface) for a library amplification (~13-cm plates). Each plate can take 20,000 to 40,000 phage; maybe 25,000 is a good number. Follow the rest of the Gigapack protocol for phage amplification.
- Allow phage plaques to grow for 7 to 8 h until they are pinhead sized (not too large).
- Overlay the plates with 9 to 10 mL of SM buffer overnight. It is very important that the plates lie very flat so that there is liquid on the whole plate into which the phage can elute. If the plates don't lie flat, parts will dry up as the buffer soaks into the agar, thus causing some uneven amplifications of phage populations.
- Recover and pool about 7 mL of SM-buffer/plate into 1-liter sterile bottles.
- Remove the bacteria with chloroform as described in the protocol, and aliquot the phage suspension into sterile 50-mL Corex tubes for centrifugation. Freeze most of the amplified libraries as aliquots at -70°C with some chloroform/DMSO as described.
- Test both primary and secondary libraries for blue/clear phage indicating ratios of phage with no cDNA insert to those with an insert. Those without an insert should have the *LacZ* gene intact and thus produce β -galactosidase that in the presence of inducer (IPTG) and substrate X-gal would form blue plaques. Phage with insert are clear.
- Mix 1 μL of unamplified phage or 5 to 8 μL of a 10^5 -dilution of amplified library with 200 μL of bacteria to get 200 to 300 phage plaques per plate.
- Incubate the phage and bacteria for ~20 min at 37°C , quickly mix with 3.0 mL of 49°C top agarose, 7.5 μL of 1 M IPTG, and 50 μL of a solution of 250 mg of X-gal per mL of dimethylformamide (DMF), and pour on a prewarmed LB plate.
- Allow the top agarose to harden without a lid for 5 min, then incubate the plate upside down overnight at 37°C .
- Remove the plates the following day and place them at 4°C for the day (or alternatively longer) for the blue color to fully develop. The primary libraries have 2 to 3% of phage that are blue (dark or light blue) indicating no insert. The rest should contain inserts. Amplified libraries are only 3 to 5% nonrecombinant.
- For initial titering of primary libraries, 1 μL of 10-fold dilutions is enough to use (see above).
- Dilute amplified libraries in steps of 1:100, where 10 μL of 10^{-6} , 10^{-7} , 10^{-8} (and possibly more) are plated and a range of about 800 down to 50 phage are seen on the increasingly diluted plates.

Examples of titres achieved:

FCR3S1 library: $\sim 3^9$ to 10^9 pfu/mL; TM 284 was at $\sim 9^9$ to 10^9 pfu/mL.

Libraries were aliquoted as 1-mL samples in microfuge tubes and stored with 50 μL of chloroform, along with some larger aliquots stored in screw-cap glass tubes with some chloroform at 4°C for library screening. The rest were kept for long-term storage as aliquots in 15-mL tubes with DMSO/chloroform at -70°C (see also the Gigapack protocol).

Testing excision of inserts from libraries

To get an idea of insert sizes in the libraries, we used recombinant phage directly from the libraries to excise, or release, clones as phagemids. Best results were obtained with

pExCell release directly from the library as opposed to plaque-purified phage clones. For the in vivo excision, we used NP66 *E. coli* accompanying the kit as well as the specified medium and temperature shift from 32 to 39 °C (See Pharmacia λExCell protocol).

- Pick colonies the following day.
- Incubate them in LB-amp overnight.
- Perform plasmid mini-preps using the alkaline lysis method from “Current Protocols” with suggested modifications, such as phenol extraction, etc. (Do not use boiling mini-preps.)
- Use *PvuII* enzyme to cut around insert sites. Use about half of the digest on a gel. *PvuII*-cut sites are ~380 bp apart on the vector (i.e., without insert).
- The vector arms should appear as one band of ~2.5 kb. Insert sizes from a few random clones tested were seen from ~0.5 to 2.2 kb. Or simply test insert size by PCR using vector arm primers.

References

Protocols from the following companies' kits:

5Prime → 3Prime's Maxi oligo (dT) Cellulose spin column for mRNA purification
Pharmacia's Timesaver cDNA Synthesis
Pharmacia's Directional Cloning Toolbox
Pharmacia's λ ExCell NotI/EcoRI/CIP
Stratagene's Gigapack Packaging Extracts
Stratagene's picoBlue Immunoscreening

Ausubel FM, et al., eds. Current protocols in molecular biology. New York: Wiley-Interscience; 1988-1999.

IV. PCR of AT-rich genomes/regions

by **Martha Schlichtherle** and **Mats Wahlgren**

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden

e-mail: mats.wahlgren@mtc.ki.se

Noncoding regions of *Plasmodium falciparum* tend to be extremely rich in AT content (>90%). PCR amplification of these regions is significantly improved by lowering the *Taq* polymerase extension temperature from 72 to 60 °C. The other parameters do not need to be changed.

Note: The AT content of the entire genome is estimated to be ~82% while coding regions are thought to be 60 to 70% AT.

References

Bowman S, Lawson D, Basham D, Brown D, Chillingworth T, Churcher CM, Craig A, Davies RM, Devlin K, Feltwell T, et al. 1999. The complete nucleotide sequence of chromosome 3 of *Plasmodium falciparum*. *Nature* 400(6744):532-538.

Su XZ, Wu Y, Sifri CD, Wellems TE. 1996. Reduced extension temperatures required for PCR amplification of extremely A+T-rich DNA. *Nucleic Acids Res* 24(8):1574-1575.

V. Single cell RT-PCR of *var* (DBL-1) mRNA from *Plasmodium falciparum*

by **Victor Fernandez**

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: victor.fernandez@smi.ki.se

Equipment

PCR machine with hot lid

Materials and reagents

GeneAmp RNA PCR Kit (Applied Biosystems)

RNase-free DNase, 10 U/ μ L (Stratagene)

DNase

RNase inhibitor

Opti-Prime PCR buffer 3 and buffer 4, mixed 1:1 (Stratagene)

TaqStart antibody (Clontech)

Taq DNA polymerase, 5000 U/ μ L (Pharmacia)

- Prepare PCR mix for n samples:

deoxyadenosine triphosphate:	$2 \mu\text{L} \times n$
deoxycytidine triphosphate:	$2 \mu\text{L} \times n$
deoxyguanosine triphosphate:	$2 \mu\text{L} \times n$
deoxythymidine triphosphate:	$2 \mu\text{L} \times n$
GeneAmp 10 $\times$ PCR Buffer II:	$2 \mu\text{L} \times n$
MgCl ₂	$4 \mu\text{L} \times n$
distilled water:	$1 \mu\text{L} \times n$
- Aliquot the PCR mix into n thin-walled PCR tubes (15 μ L/tube).
- Place a single infected erythrocyte into each tube and immediately freeze it on dry-ice (see PARASITES, section V:B).
- Release the DNA and RNA from the parasite by heating the tubes to 93 °C for 3 min in the PCR machine. Cool them on ice.
- Add 1 μ L of DNase and 1 μ L of RNase inhibitor to each tube. Degrade the DNA at 37 °C for 30 min in the PCR machine. Inactivate the DNase at 93 °C for 3 min.
- Add 1 μ L of RNase inhibitor, 1 μ L of random hexamers, and 1 μ L of reverse transcriptase to each tube. Perform the reverse transcription at 42 °C for 30 min and inactivate the reverse transcriptase at 93 °C for 3 min.
- Prepare the TaqStart mix for n reactions:

Taq polymerase:	$4.4 \mu\text{L} \times m$ (where $m = 4.8 \times n / 25.4$)
TaqStart antibody:	$4.4 \mu\text{L} \times m$
TaqStart buffer:	$17.6 \mu\text{L} \times m$

 Mix and incubate for at least 5 min at room temperature.
- Prepare the PCR master mix for n reactions:

Opti-Prime PCR buffer 3+4:	$8 \mu\text{L} \times n$
Specific primer DBL1-1 (DBL1-3):	$2 \mu\text{L} \times n$
Specific primer DBL1-2:	$2 \mu\text{L} \times n$
TaqStart mix:	$4.8 \mu\text{L} \times n$
distilled water:	$63.2 \mu\text{L} \times n$

- Add 80 μL of PCR master mix to each tube. The tubes already contain 20 μL from the previous steps; the final volume in each tube should thus be 100 μL .
- Amplify the *var* sequences using the following PCR conditions:
93° C for 20 s, 55 °C for 30 s, and 72 °C for 1 min.
Run 50 cycles followed by a last extension step at 72 °C for 7 min.
- Run 20 μL of the PCR products on a 1.2% agarose gel with TBE buffer.

Primer sequences

DBL1-1: 5'-GGW GCW TGY GCW CCW TWY MG-3'

DBL1-2: 5'-ARR TAY TGW GGW ACR TAR TC-3'

DBL1-3: 5'-GCA CGA AGT TTY GCA GA-3'

Reference

Chen Q, Fernandez V, Sundström A, Schlichtherle M, Datta S, Hagblom P, Wahlgren M. 1998. Developmental selection of *var* gene expression in *Plasmodium falciparum*. Nature 394(6691):392-395.

VI. Fluorescent in situ hybridization (FISH)^{DNA-DNA} for *Plasmodium falciparum*

(Adapted from Ersfeld, K. & Gull, K, 1997)

by **Lucio Freitas-Junior** and **Artur Scherf**

Institut Pasteur, Unité BIHP, 25 rue du Dr. Roux, 75015 Paris, France

e-mail: junior@pasteur.fr, ascherf@pasteur.fr

Equipment

thermal cycler with in situ adapter (Eppendorf)
water baths (37 °C, 50 °C, and 60 °C)
humid chamber
microscope that allows analysis of different fluorochromes, CCD camera attached (e.g., Nikon 800)

Materials and reagents:

saponin
incomplete RPMI
RPMI 1640
25 mM HEPES
0.37 mM hypoxanthine (pH 7.4)
standard microscope slides
In situ Frame for 25 µL (Eppendorf)
hybridization solution (HS):
50% formamide (Roche Applied Science)
10% dextran sulfate
2× SSPE
250 µg/mL herring sperm DNA
Probes: To prepare the probes, use the “Fluorescein High-prime kit” (Roche Applied Science) for Fluorescein signals and “Biotin high-prime kit” (Roche Applied Science). For detection of the biotinylated probes use avidin-conjugate with rhodamine (Roche Applied Science).
M solution (pH 7.5):
100 mM maleic acid
150 mM NaCl
1% blocking reagent (Roche Applied Science)
4% bovine serum albumin (BSA) (SIGMA)

TNT solution (pH 7.5):
100 mM Tris-HCl
150 mM NaCl
0.5% Tween 20 (v/v)

20× SSC
0.1% NP 40 in PBS
2% paraformaldehyde in PBS (pH 7.2)
formamide (Roche Applied Science)
50%, 70%, 90%, and 100% ethanol
VECTASHIELD mounting medium with DAPI (Vector Laboratories)

Procedure

Parasite fixation:

Before starting, mark the region where the parasites will be deposited on the slide with a diamond marker: This makes it easier to visualize them later. This area should be about 5 mm × 5 mm.

- Treat the parasites with saponin to lyse the erythrocyte membrane.
- Wash them twice in incomplete RPMI.
- Deposit a monolayer of parasites in the area previously marked on the microscope slides.
- Air dry the slides for 30 min (or overnight) at room temperature.
- Fix the slides in 2% paraformaldehyde in PBS (pH 7.2) for 5 min.
- Wash the slides three times in PBS and dehydrate them through a series of ethanol washes (50%, 70%, 90%, and 100%) for 5 min each.
- Air dry the slides for 30 min. At this point you can use the slides or store them at –80 °C (in aluminium foil).

In situ hybridization:

- Remove the slides from –80 °C just before use and leave them at room temperature for 10 min before removing the aluminium foil.
- Permeabilize the parasites by incubating them in 0.1% NP 40 in PBS for 5 min at room temperature.
- Wash the slides 3 times in PBS for 5 min each at room temperature.
- Fix the plastic frame around the area containing the parasites.
- Apply the HS solution (denatured at 95 °C for 5 min) on this area.
- Incubate the slides for 30 min at 42 °C in a humid chamber.
- For hybridization, remove as much of the HS solution as possible and add fresh HS containing the probes. (For biotin-labeled probes, use a final concentration of 1 ng/μL and for fluorescein probes use a final concentration of 0.5 ng/μL.)
- Cover the area with the In situ Frame coverslip.
- Denature the slides in a thermal cycler with in situ adapter for 2 min at 94 °C followed by hybridization at 37 °C overnight.

Washing:

- Remove the In situ Frame coverslip and the HS solution.
- Wash the slides in 2× SSC/50% formamide for 30 min at 37 °C, followed by 2× SSC for 10 min at 50 °C, 2× SSC for 10 min at 60 °C, and 4× SSC for 10 min at room temperature.
- Equilibrate the cells in M solution for 5 min at room temperature in a humid chamber protected from light.
- Remove the M solution and replace it with M solution plus avidin–rhodamine (final concentration: 50 ng/mL) for detection using biotin probes. Incubate the slides for 30 min at room temperature.
- Wash the slides in M solution for 10 min at room temperature, and twice in TNT solution for an additional 10 min each at room temperature.
- Dry the slide and mount using Vectarshield with DAPI.
- Analyse the slide by fluorescence microscopy.

Comments

Always wear gloves!

In optimal conditions the FISH technique will give a clean result without any background.

Never let the slides dry out after the hybridization process has begun.

This technique works nicely for other *Plasmodium* species and also works for *Leishmania* however for *Leishmania* the fixation is performed in 100% ethanol for 10 min at room temperature.

References

Ersfeld K, Gull K. 1997. Partitioning of large and minichromosomes in *Trypanosoma brucei*. Science 276(5312):611-614.

Freitas-Junior LH, Bottius E, Pirrit LA, Deitsch KW, Scheidig C, Guinet F, Nehrbass U, Wellem TE, Scherf A. 2000. Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of *P. falciparum*. Nature 407(6807):1018-1022

VII. Separation and mapping of chromosomes of malaria parasites using pulsed-field gel electrophoresis (PFGE)

VII.A. PFGE Protocol 1

by **Rosaura Hernandez-Rivas¹** and **Artur Scherf²**

¹Department of Molecular Biomedicine, Centro de Investigación y de Estudios Avanzados del I.P.N., México D. F., México

e-mail: rosaura36@hotmail.com

²Institut Pasteur, Unité BIHP, rue du Dr. Roux, 75015 Paris, France

e-mail: ascherf@pasteur.fr

Equipment

Pulsaphor/Gene Navigator PFGE apparatus (Pharmacia) or CHEF-DR system (Bio-Rad)
centrifuge
water bath
15-mL Falcon tubes (BD Labware)

Materials and reagents

SeaKem GTG agarose (Cambrex) for agarose gels at a concentration of >0.6%
chromosomal grade agarose (Bio-Rad) for agarose gels ≤0.6%
low-melting point agarose (Cambrex) for embedding parasites in agarose blocks and preparative isolation of chromosomes
plastic gel molds (2 × 5 × 10 mm, 100-μL volume; Pharmacia)
nylon membrane: Hybond-N+ (Amersham) or Biotrans(+) (ICN Biomedicals)
These membranes allow the rapid alkaline transfer of DNA and their physical characteristics make them especially useful for multiple hybridization cycles (usually 5 to 10 cycles).
erythrocyte lysis buffer: 0.15% saponin (Sigma) in phosphate-buffered saline (PBS).
ethidium bromide in water: 10 mg/mL
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

alkaline transfer buffer: 0.4 M NaOH

20× SSC:

3 M sodium chloride

0.3 M sodium citrate

TE buffer:

10 mM Tris-HCl

1 mM EDTA (pH 8.0)

1 mM phenylmethylsulphonyl fluoride (PMSF) in TE

molecular weight size standards:

5 kb ladder (4.9 to 120 kb)

ladder (0.05 to 1 Mb)

Saccharomyces cerevisiae (0.2 to 2.2 Mb)

Hansenula wingei (1 to 3.1 Mb) (Bio-Rad)

cell lysis buffer (CLB):

0.5 M EDTA (pH 8.0)

10 mM Tris-HCl

1% sodium lauryl sarcosinate (Sigma)

Store at room temperature. Before cell lysis, add 2 mg/mL of proteinase K from a stock solution.

prehybridization and hybridization buffer:

0.5 M NaPO₄ (pH 7.2)

7% SDS

1% BSA

hybridization buffer:

7% SDS

0.5 M EDTA (pH 8.0)

1% BSA

saturated 2-butanol:

Mix 2-butanol with 1 M NaCl in TE (4:1 v/v). Add 9 volumes of saturated 2-butanol and 1 volume of the aqueous phase to the gel blocks. This is important to avoid gel shrinking!

10× TBE buffer:

108 g Tris-base

54 g boric acid

8.35 g disodium-EDTA per liter (pH ~8.5)

Preparation of DNA for PFGE

The ability to preserve the intact size of large DNA molecules is critical for the success of PFGE. To overcome the problem of DNA shearing, intact cells are embedded in low-melting agarose blocks. Cells are lysed and proteins are removed by proteinase K treatment. This procedure yields DNA that is both intact and susceptible to restriction enzyme digestion. The agarose block can be loaded directly into the well of a pulsed-field gel.

The host cells of the intracellular blood stage form of *Plasmodium falciparum* need to be lysed by saponin before the parasite cells are embedded into agarose blocks.

- Estimate the volume of the erythrocyte pellet and add 1.5 volumes of 0.15% saponin in PBS at room temperature.
- Resuspend the pellet and incubate it for 3 to 5 min. The liquid will clarify as the red blood cells lyse.
- Add 5 volumes of cold PBS and centrifuge the pellet at 5,000 rpm for 10 min.
- Resuspend the dark pellet which contains the parasite material in 2 mL of PBS.
- Pellet the cells by centrifugation at 3,000 rpm for 10 min.
- Resuspend the pellet at a concentration of approximately 5×10^8 parasites/mL of PBS.
- Equilibrate the cell suspension at 37 °C and add an equal volume of 1.6% melted low-melting point agarose (37 °C).
- Mix the cell suspension gently and dispense it immediately into plastic gel molds.
- Allow the gel to solidify at 4 °C for about 20 min.

- Place up to 20 solidified blocks in 10 mL of cell lysis buffer (CLB) and incubate them for 24 h at 42 °C.
- Replace the CLB with an equal volume of fresh CLB.
- Incubate the blocks for an additional 24 h.
- The blocks can be stored indefinitely in TE buffer at 4 °C.

Electrophoresis conditions

Optimal separation of the different chromosome size classes usually requires the use of a range of different PFGE conditions. Table 1 shows the running conditions of large DNA fragments of various sizes using the CHEF (clamped homogeneous electric field) system. These PFGE methods have been used in studies of *Plasmodium falciparum* and *Trypanosoma cruzi*, but also apply to other parasitic protozoa. The described electrophoresis conditions have been developed for the Pulsaphor/Gene Navigator PFGE apparatus (Pharmacia) based on the CHEF design. Similar results have been obtained with the CHEF-DR system (Bio-Rad).

Table 1. Electrophoresis conditions for separation of DNA fragments by PFGE (CHEF system)

Efficient separation	Pulse time (ramping)	Voltage	Run time	Agarose concentration	Buffer
5–100 kb	0.5 s – 1.0 s	400 V	7 h	1.3%	0.2× TBE
50–2000 kb	50 s – 90 s	200 V	22 h	1.0%	0.5× TBE
500–4000 kb	90 s – 300 s for 24 h followed by 300 s – 720 s for 24 h	95 V	48 h	0.7–0.8%	0.5× TBE
		85 V			
2000–6000 kb	120 s – 720 s	85 V	36 h	0.4–0.6% high gel strength agarose	0.5× TBE

- The agarose concentration and type of agarose used depends on the DNA size range to be separated. For example, pulsed field separations larger than 2 megabases are improved by using high gel-strength agarose (chromosomal grade agarose, Bio-Rad) allowing preparations of very low-percentage agarose gels (0.4 to 0.6%), which give significantly better separation of large chromosomes in the range of 2 to 5 megabases..
- For a 15 cm × 15 cm × 0.5 cm 1% agarose gel, dissolve 1.1 g of SeaKem GTG agarose in 110 mL of 0.5× TBE buffer by heating it in a microwave oven. Make sure that the volume has not changed. If it has, adjust again to 110 mL.
- Cool the agarose in a 50 °C water bath and pour it into the casting stand.
- After the gel is solidified, fill the wells with 0.5× TBE buffer and insert the blocks containing parasite DNA. About 2 to 5 mm of a block (1×10^7 to 2.5×10^7 parasites) generally contains enough material to visualize the chromosomes after ethidium bromide staining. (This number may also work for other parasite

species.) However, for best PFGE results it may be necessary to determine the optimal number of cells/block.

- Seal the blocks into the well with 1% low-melting point agarose.
- Fill the electrophoresis chamber with 2.2 liters of TBE buffer. Do not overfill; the buffer should just cover the top of the agarose gel. Keep the temperature of the running buffer (18 °C) constant during the run.

Staining of chromosomes

- After completing the PFGE run, the separated chromosomes can be visualized by staining the agarose gel for 15 min in ethidium bromide (1 µg/mL in water). Destain the gel by two washes in 0.5× TBE for 1 h with gentle agitation, and photograph it (do not forget the ruler) using a shortwave UV light (254 nm). **ATTENTION!** If chromosomes are intended for use in subsequent restriction mapping studies, longwave UV light (360 nm) must be used to avoid nicking the DNA.

Southern hybridization

- Transfer the DNA from the agarose gel to a nylon membrane such as Hybond-N or Biotrans. In order to ensure efficient transfer of large DNA fragments, nick the chromosomal DNA by exposing the stained agarose gel for 5 min on a UV-light table (254 nm).
- The alkaline transfer procedure using 0.4 N NaOH as transfer buffer works fine for chromosomal DNA blots. We routinely set up the capillary transfer (standard molecular biology procedure) for at least 24 h. Make sure that the weight on top of the absorbent paper stack does not exceed 1 kg. There is no need to fix DNA after alkali blotting.
- For ³²P-labelled DNA probes, the random hexamer priming method in combination with the following hybridization buffer usually works well to give a good signal-to-background ratio with nylon membranes: 7% SDS, 0.5 M EDTA (pH 8.0), 1% BSA. Single-copy genes of *P. falciparum* are generally detected after 6 h to overnight exposure on X-ray film.
- Remove probes by standard protocols or as recommended by the manufacturer. Stripped blots can be stored for prolonged periods at room temperature.

Restriction enzyme digestion of embedded chromosomes

Generally, restriction enzymes diffuse into agarose blocks and are thus suited for chromosome mapping studies. The most useful restriction endonucleases are those with 8-base recognition sites which cut only a few times in chromosome-sized DNA fragments. However, in genomes which are biased in their AT content, such as *P. falciparum*, certain enzymes which recognize GC-rich 6-base pair sequences (*Sma*I, *Bgl*I) can also be used as “rare” cutters in these AT-rich genomes.

- For restriction enzyme digestion of agarose-embedded total parasite DNA, remove the lysis buffer by extensive washes in TE buffer (2 to 3 washes in 20 mL of TE).
- Inactivate any remaining proteinase K by treating the blocks with 1 mM phenylmethylsulphonyl fluoride (PMSF) in TE (10 mL for 20 blocks) for 2 h at room temperature, followed by three washes in 10 mL of TE for 30 min each.

Individual chromosomes or DNA fragments that have been cut out of a stained agarose gel after a PFGE run must be treated with 2-butanol to extract any remaining ethidium bromide prior to restriction enzyme digestion.

- Add 9 volumes of 2-butanol saturated in 1 M NaCl/TE and 1 volume of the aqueous phase to the gel block (5:1, v/v).
- Agitate the mixture at room temperature for 30 min.
- Repeat the extraction twice and wash the block five times in 10 mL of TE buffer for 30 min each.
- Store the blocks at 4 °C.
- Equilibrate the blocks with 5 volumes of restriction buffer containing 100 µg/mL of nuclease-free BSA for 30 to 60 min at room temperature. Remove the buffer and add 2 volumes of fresh buffer and restriction endonuclease (approximately 100 to 200 U/mL). Incubate the blocks at the recommended temperature for 4 h or overnight.
- Partial digestion of chromosomal DNA is useful for mapping studies. In this case, set up separate digestions of serial dilutions of the restriction enzyme for 2 h each (0.01 U/mL, 0.1 U/mL, 1 U/mL, 10 U/mL, and 100 U/mL. Stop the reactions by adding EDTA to a final concentration of 50 mM.
- Depending on the expected fragment size, restricted DNA can be separated by PFGE according to the run conditions described in Table 1.

Two dimensional PFGE

Most chromosomes in *P. falciparum*, as in other protozoan parasites, are heterogeneous in size and can be separated from each other by PFGE. Two-dimensional (2D) PFGE studies of a karyotype can give useful information concerning genetic markers located on several chromosomes. Multigene families are often observed in pathogenic protozoa.

- Separate chromosomes in the first dimension and stain them with ethidium bromide as described above.
- Photograph the gel using longwave UV light (360 nm).
- Use a razor blade to excise strips of gel (~2 mm) that contain all chromosomes.
- Extract ethidium bromide using 2-butanol in a 15-mL Falcon tube (as described above).
- Perform restriction enzyme digestions as described above.
- Embed the slice of gel containing the digested chromosomes and size markers (λ ladder or 5 kb ladder) into a precut slot in the top of a 1.3% agarose gel and seal it with 1% low-melting point agarose.
- Carry out standard PFGE separation using the run conditions described in Table 1. For Southern hybridization, continue as described above.

References

Hernandez-Rivas R, Scherf A. 1997. Separation and mapping of chromosomes of parasitic protozoa. Mem Inst Oswaldo Cruz 92(6):815-819.

Hinterberg K, Scherf A. 1994. PFGE: improved conditions for rapid and high-resolution separation of *Plasmodium falciparum* chromosomes. Parasitol Today 10(6):225.

VII:B. PFGE Protocol 2by **Mats Wahlgren***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: mats.wahlgren@mtc.ki.se*Equipment

centrifuge
incubator
Plug mold (Bio-Rad)
PosiBlot 30-30 pressure blotter (Stratagene)
Stratalinker UV crosslinker (Stratagene, model 2400)
Falcon tubes (BD Labware)

Materials and reagents

10% saponin in PBS
10 mM EDTA (pH 7.6)
PBS
low-melt agarose
0.5 M EDTA, 1% *N*-lauryl sodium sarcosinate (sarkosyl), proteinase K
chromosomal grade agarose (Bio-Rad)
ethidium bromide
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

0.25 M HCl
denaturation buffer:
0.5 M NaOH
1.5 M NaCl
neutralization buffer:
1 M Tris
1.5 M NaCl
10× SSC
nylon membrane Hybond-N (Amersham)

TSE:
10 mM Tris-HCl (pH 8.0)
100 mM NaCl
1 mM EDTA

Sample preparation

- Calculate the parasitemia of the culture to be used.
- Add 1/100 volume of 10% saponin to the culture. Incubate the culture for 5 min at room temperature.
- Pellet the free parasites by centrifuging them at ~1,000 rpm for 5 min (i.e., a “gentle spin”).
- Dissolve the pellet in PBS and spin it again. When dissolving the pellet, use a broad tip.
- Wash the pellet two more times with PBS.

- After the last centrifugation, dissolve the pellet in TSE to get a concentration of 8×10^8 parasites/mL.
- Mix the parasites with an equal volume of 1.6% low-melt agarose to get a final concentration of 4×10^8 parasites/mL. The agarose should be between 37 and 42 °C.
- Dispense the mix into plug molds and let the agarose solidify, first at room temperature and then at 4 °C for 20 to 30 min.
- Put the DNA/agarose plugs in 0.5 M EDTA, 1% *N*-lauryl sodium sarcosinate, 2 mg/mL of proteinase K; 10 mL of solution will be enough for 20 DNA plugs in a Falcon tube.
- Incubate the plugs at 37 °C for 48 h.
- Store the DNA plugs at 4 °C in the same solution as above (or possibly in TE).

Electrophoresis conditions

See also the section on electrophoresis conditions in *MOLECULAR BIOLOGY*, section VII:A, for details.

Separation		Pulse time (ramping)	Voltage	Run time	Included angle	Buffer/ agarose *
Quick separation 500–2500 kb	Blk 1	5–12 min	2.0 V/cm	30 h	53°	1× TAE/ 0.7%
	Blk 2	3–5 min	4.0 V/cm	17.5 h	53°	
Good separation 500–1800 kb	Blk 1	6–8 min	2.5 V/cm	80 h	60°	0.5× TBE/ 1%
	Blk 2	4–6 min	2.5 V/cm	64 h	60°	
	Blk 3	2–4 min	2.5 V/cm	20 h	60°	

* The agarose used is chromosomal grade agarose (Bio-Rad). Buffer temperature is 14 °C for quick separation and 10 °C for good separation.

Southern blot

- Stain the gel for 30 min in 1 µg/mL of ethidium bromide. Destain the gel for 10 min in distilled water. Look at the gel under UV light and take a photograph. Destain the gel for another hour in distilled water.
- Prepare the gel for Southern transfer by depurinating it in 0.25 M HCl for 30 min.
- Rinse the gel in distilled water and denature it in denaturation buffer for 30 min.
- Rinse the gel in distilled water and neutralize it in neutralization buffer for 30 min.
- Rinse the gel in 10× SSC. Wet the nylon membrane in distilled water and let it soak for 5 min in 10× SSC.
- Transfer the chromosomes from the gel to the nylon membrane in a PosiBlot 30-30 pressure blotter. Assemble the pressure blotter according to the manual and use 10× SSC as the transfer buffer. One hour at 75 to 80 mm Hg is enough to transfer the chromosomes to the membrane.
- Quickly rinse the filter in 2× SSC to get rid of any gel pieces sticking to the membrane. Put the membrane on a Whatman paper to dry off a little.

- While the membrane is still moist, cross-link the DNA to the membrane by exposing it to UV light in a crosslinker. Use setting “auto cross-link”.

References

Chen Q, Fernandez V, Sundström A, Schlichtherle M, Datta S, Hagblom P, Wahlgren M. 1998. Developmental selection of *var* gene expression in *Plasmodium falciparum*. *Nature* 394(6691):392-395.

Dolan S, Adam R, Wellems T. 1993. Chromosome mapping methods for parasitic protozoa. *In*: Hyde JE, ed. *Methods in molecular biology*. Vol 21. Totowa, NJ, USA: Humana Press; p 319-332.

VIII. Recombinant expression of *Plasmodium falciparum* var- gene domains

by Qijun Chen

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm Sweden

e-mail: chen.qijun@smi.ki.se

Equipment

bacterial culture flask
bacterial culture incubator
sonicator/French press
centrifuge (4 °C)
spectrophotometer

Materials and reagents

pGEX vector (Pharmacia)
BL21-CodonPlus-RIL competent cells (Stratagene) or BL21 competent cells (Pharmacia Biotech)
LB plates (ampicillin 100 µg/mL)
PBS (pH 7.2)
PBS plus 0.2 M NaCl and 0.05% Tween-20
2× YT/amp medium:
 16 g/L tryptone
 10 g/L yeast extract
 5 g/L NaCl
 ampicillin 100 µL/mL
EDTA
PTG (1 M)
Pefabloc (Roche Applied Science)
lysozyme (Pharmacia Biotech)
Triton X-100
glutathione-Sepharose (Pharmacia Biotech)
glutathione solution
thrombin (Pharmacia Biotech)

Procedure

- Clone the sequence of var-domains into the pGEX vector in the correct reading frame.
- Transform BL21-CodonPlus-RIL competent cells or BL21 competent cells and select the positive transformed bacteria with ampicillin in LB (100 µg/mL) using manufacturer's conditions. The former strain appears to result in less degradation during expression.
- Pick several colonies and culture them separately overnight in 20 mL of LB medium with 100 µg/mL of ampicillin at 37 °C.
- Inoculate the overnight culture in prewarmed (room-temperature) 2× YT/amp medium in a ratio of 1:100. Use 20 mL of medium for the initial expression test, and 1 to 10 liters for large scale expression. Continue shaking the culture at room temperature until the OD₆₀₀ of the culture is 0.4 to 0.6. Precool the centrifuge to 4 °C.

- Check the induction system for your vector. If you are using the pGEX system and thus the *tac*-promoter, add IPTG to 0.1 mM (using a 1 M stock) and induce the expression at a temperature lower than 30 °C:
overnight for the ATS-region
less than 4 h for DBL-domains
- Sediment the bacteria by centrifugation at $1,500 \times g$ for 20 min at 4 °C if using large-volume centrifuge flasks. For small volumes, sediment for 10 min.
- Resuspend the bacteria with cold PBS (20 times the pellet volume), then add:
EDTA to 0.5 mM (do NOT use if using a his-tag vector)
Pefabloc to 1 mM (or 1 mg/mL)
lysozyme to 1 mg/mL
(DTT to 5 mM, optional)
- Mix completely and sonicate the bacteria five times for 18 s each, or use a French press to release the fusion protein from the bacteria. You may have to pass the bacterial solution through the press twice to release protein.
- Add 20% Triton X-100 to a final concentration of 1% and shake it at 4 °C for 30 min. If using a GST-vector, prepare glutathione–Sepharose according to manufacturer's instructions and follow the protocol below. Otherwise, refer to the manufacturer's recommendations for the expression system you have chosen.
- Centrifuge bacterial lysis at $2,000 \times g$ for 15 min at 4 °C.
- Remove and save the supernatant, add to it at least 0.5 mL of bead volume of glutathione–Sepharose per liter of original culture.
- Allow the binding of the fusion protein and matrix to proceed for 1 h with gentle shaking at 4 °C.
- Wash the adsorbed beads twice with cold PBS plus 0.2 M NaCl and 0.05% Tween-20 and once with cold PBS only.
- Sediment the suspension by centrifuging ($2,000 \times g$). Remove the supernatant.
- Add an equal volume of glutathione solution to the beads and release the protein for 60 min at room temperature, or overnight at 4 °C with slow shaking.
- Sediment the beads and measure the yield of fusion protein in the supernatant with the spectrophotometer at 280 nm.
- The fusion protein can be digested either directly on the glutathione–Sepharose beads or in the eluent solution. Use 30 U of thrombin per 500 μ L of bead volume of beads or 1 mL of eluent solution. The digestion is usually carried out overnight at 4 °C.
- Aliquot, then store the fusion protein at –70 °C.

Reference

Chen Q, Heddi A, Barragan A, Fernandez V, Pearce FA, Wahlgren M. 2000. The semiconserved head structure of *Plasmodium falciparum* erythrocyte membrane protein 1 mediates binding to multiple independent host receptors. *J Exp Med* 192(1):1-9

IX. Transfection of *Plasmodium falciparum* within human red blood cells

by Yimin Wu

Malaria Vaccine Development Unit, NIAID, National Institutes of Health,
5640 Fishers Lane, Rockville, MD 20852, USA

e-mail: yiwu@niaid.nih.gov

There are 3 basic steps in asexual stage *P. falciparum* transfection:

- A. Preparing the materials, parasite culture, and DNA constructs
- B. Transfecting DNA into parasites by electroporation
- C. Assaying the expression of transfected genes

This protocol includes the chloramphenicol acetyl-transferase (CAT) assay, luciferase assay, and the selection of stable transformants by the anti-folate pyrimethamine.

IX:A. Materials

Parasite culture

Prepare 1×10^9 to 2×10^9 parasitized RBCs (PRBCs) for each transfection. A synchronized ring-stage parasite (<20 h) culture with high parasitemia (>15%) is essential for an improved transfection efficiency. For cultivation, synchronization, and purification see PARASITES, sections I through IV. Because washed human RBCs deteriorate after 4 weeks at 4 °C (even sooner at 37 °C), the parasite culture should be replenished with fresh RBCs before transfection, especially when a stable selection is desired.

DNA constructs

The design of transfection constructs is specific to the goal of each individual experiment. Plasmodial flanking regions are required for the expression in parasites; see also “References” at the end of this protocol. Use 50 to 100 µg of DNA for each transfection. The plasmid may be dissolved in TE buffer at 1 to 3 mg/mL.

Buffers

Incomplete Cytomix:

- 120 mM KCl
- 0.15 mM CaCl₂
- 2 mM EGTA
- 5 mM MgCl₂
- 10 mM K₂HPO₄/KH₂PO₄
- 25 mM HEPES

PBS:

- 1.7 mM NaH₂PO₄
- 5 mM Na₂HPO₄
- 150 mM NaCl
- 10% saponin/PBS stock solution

Equipment

- GenePulser (Bio-Rad)
- Capacitance Extender (Bio-Rad)
- electroporation cuvettes (0.2-cm and 0.4-cm gap)
- table-top centrifuge (Sorvall)

Reagents, buffers, and equipment for CAT assay

TEN:

1 mM EDTA

150 mM NaCl

40 mM Tris–HCl (pH 7.6)

250 mM Tris–HCl (pH 7.6)

¹⁴C]chloramphenicol (110 mCi/mmol, Moravsek Biochemicals, diluted to 2.5 nCi/μL)

acetyl coenzyme A (20 mg, Calbiochem, dissolved in 250 mM Tris–HCl [pH 7.6] to 40 mM)

ethyl acetate

chloroform

methanol

TLC plate (aluminium-backed, thin-layer silica gel 60A plates, Whatman)

glass chromatography chamber

X-ray film and film cassettes, alternatively Phosphorimager cassettes and scanner (Molecular Dynamics)

Reagents, buffers, and equipment for luciferase assay

Luciferase Assay System (Promega):

cell lysis buffer

luciferase substrate

substrate solvent

purified luciferase as positive control

luminometer (Turner)

Reagents for stable selection

Pyrimethamine stock solution is made in dimethyl sulfoxide (DMSO, 50 mg/mL).

Make the selection culture medium without gentamycin. The final content of DMSO in the culture medium should be less than 0.5%.

IX:B. TransfectionTransfecting parasites, Day 1

- Change the parasite culture supernatant with fresh medium and make a smear to ensure the quality of the culture. Return the culture to the incubator for at least one h while preparing for transfection.
- Chill the electroporation cuvette and the Cytomix solution on ice. Set the GenePulser electroporator at 2.5 kV, 25 μF, 200 Ω.
- After everything is in place, spin down the cells at 1,500 rpm for 5 min in a table-top centrifuge. Resuspend the cells (about 100 to 200 μL) with 500 μL of Cytomix and not more than 100 μL of the DNA construct*. Mix well by pipetting up and down several times.

*Note: The DNA construct solution should be further concentrated if the volume to be added exceeds 100 μL in order to reach the desired final concentration in the transfection mix.

- Transfer the mixture into a 0.4-cm cuvette and execute the electroporation. Take a note of the set voltage, the actual voltage, capacitance, and time constant. A

slight deviation of actual voltage is acceptable. The time constant should be 0.7 to 0.9 ms.

- Immediately return the cuvette to ice for 5 min, then transfer the cells from the cuvette to a culture flask together with 10 mL of culture medium (rinse the cuvette with culture medium to ensure a complete transfer). Return the flask to the incubator.
- An alternative electroporation setting is 0.31 kV/960 μ F with the Bio-Rad GenePulser, using a 0.2-cm cuvette. The total volume allowed in the 0.2-cm cuvette is 400 μ L. The time constant is about 12 to 15 ms.

Changing medium, Day 2

- Change the culture medium and add 50 μ L of fresh blood. Check the parasite smear. Don't panic if the smear contains lots of extracellular, dying parasites or cell debris, as many parasites normally die during this procedure. Recovering parasites should have developed to trophozoites or schizonts by now.

Starting selection for stable transformants or changing medium for transient expression assay, Day 3

- **For stable transformation**, this is the day to start drug pressure. Feed the culture with medium containing pyrimethamine (100 ng/mL). On *Day 4*, continue to feed the culture with medium containing pyrimethamine (100 ng/mL). Most parasites die after 2 days of drug treatment, as can be seen in a Giemsa-stained thin smear. The pyrimethamine pressure may thus be dropped to 40 ng/mL. Continue this pressure until transformants are detected in Giemsa-stained thin smear (usually 3 to 5 weeks). Add 25 μ L of fresh RBCs into the culture each week during the selection procedure.
- **For transient CAT and luciferase expression assays**, continue to change the culture supernatant with 10 mL of fresh medium. Check the smear. New rings should emerge by *Day 3*.

C. Transfectant harvest and expression assays, Day 4

- **The luciferase assay** substrate is supplied as a powder and accompanied by a vial of solvent. Once the powder is dissolved, aliquot the substrate solution and store it at -20°C . Before harvesting the cells, thaw and equilibrate the substrate to room temperature. Transfer 100 μ L of the substrate into an assay tube and cover it with a piece of aluminium foil to avoid light.
- Spin down the parasite culture, resuspend the pellet in 1 mL of PBS and transfer it into a microfuge tube. Add 10 μ L of 10% saponin/PBS stock to bring the final concentration of saponin to 0.1%; incubate the culture at room temperature for 5 min. Spin it at top speed in a microfuge for 3 min and aspirate the supernatant and RBC ghosts.
- Wash the pellet 2 or 3 times with PBS to remove hemoglobin which interferes with the luciferase assay. Resuspend the pellet completely in 30 μ L of $1\times$ lysis buffer by pipetting up and down. Freeze/thaw the sample for one cycle, then vigorously pipette up and down again. The lysis of parasites is hardly discernible by eye because a large portion of the pellet is hemozoin. Centrifuge the lysate in a microfuge for 3 min at top speed. Take 20 μ L of the supernatant for the luciferase assay.

- Because there is a slight delay of the photon peak after luciferase is added to the substrate, the timing of sample measurement is crucial if a quantitative comparison is desired. A practice optimized for Turner Luminometer measurements is to wait until the Luminometer is available (you are the only user at the time) and ready (the baseline has returned to zero). Set the timer for 10 s upon mixing the substrate and the lysate. Insert the tube into the chamber and start the measurement when the timer goes off. The set-up of the Turner Luminometer is usually done by a specialist from the vender company. This measuring protocol should be revised if a different luminometer is used.
- An alternative positive control for the luciferase assay is *Escherichia coli* cells carrying the tester plasmid.
- For the **CAT assay**, spin down the parasite culture, resuspend the pellet in 1 mL of PBS and transfer it to a microfuge tube. Add 10 μ L of 10% saponin/PBS stock to make the final concentration to 0.1% and incubate the pellet at room temperature for 5 min. Spin it at top speed in a microfuge for 3 min, then aspirate the supernatant and RBC ghosts. Wash the pellet once with 1 mL of PBS and once with 1 mL of TEN. Resuspend the cell pellet in 120 μ L of 250 mM Tris-HCl (pH 7.6) with PMSF (1 mM); pipette up and down vigorously. Freeze/thaw the sample for 3 cycles. The lysis of parasites is hardly discernible by eye because a large portion of the pellet is hemozoin. Centrifuge the lysate in a microfuge for 3 min at top speed. Incubate the lysate for 10 min at 60 °C to inactivate cellular acetylases. Clear the lysate by centrifugation in a microfuge at 15,000 $\times g$ for 2 min.
- In a microfuge tube, mix 10 μ L of lysate, 20 μ L of acetyl-CoA (40 mM), and 1 μ L of [^{14} C]chloramphenicol (2.5 nCi). Incubate the mixture at 37 °C for 3 h, then add a second 20- μ L aliquot of acetyl-CoA. Continue the incubation overnight.
- Handle all the samples, reagents, and waste according to radiation safety regulations from this point onward.

Continue the CAT assay, Day 5

- Momentarily spin down condensations of the reaction mix, then add 350 μ L of ethyl acetate. Vortex to thoroughly mix and spin briefly to separate the organic and aqueous phases. Transfer 300 μ L of the upper organic layer to a clean microfuge tube and dry the extract in a speed-vac centrifuge (30 to 60 min at 45 to 60 °C).
- Redissolve the sample with 25 μ L of ethyl acetate. Mark the origin of a thin-layer chromatography (TLC) plate (Whatman) with a soft lead pencil 1.5 to 2 cm from the edge. Apply 10 μ L of sample to the origin. Prepare a TLC chamber containing 200 mL of chloroform:methanol (20:1). Place the TLC plate in the chamber and allow the solvent front to move ~15 cm. Remove the plate from the chamber and air-dry the plate.
- Place the TLC plate in a film cassette and align the plate and the X-ray film with radioactive ink. A 3- to 5-day exposure is required due to the low level expression (or low transfection efficiency). Develop the film and align it with the plate. Alternatively, expose the TLC plate to a Phosphorimager screen for overnight and scan it with the Phosphorimager. Three radioactive spots should be observed. The two faster migrating spots represent the 1- and 3-acetylated chloramphenicol. The spot that has migrated the least distance from the origin represents the nonacetylated form.

- Liquid scintillation may be used to quantify the CAT activity. This involves cutting the radioactive spots from the plate for measurement of the amount of radioactivity they contain.

References

Crabb BS, Triglia T, Waterkeyn JG, Cowman AF. 1997. Stable transgene expression in *Plasmodium falciparum*. Mol Biochem Parasitol 90(1):131-144.

Goonewardene R, Daily J, Kaslow D, Sullivan TJ, Duffy P, Carter R, Mendis K, Wirth D. 1993. Transfection of the malaria parasite and expression of firefly luciferase. Proc Natl Acad Sci USA 90(11):5234-5236.

Mamoun CB, Gluzman IY, Goyard S, Beverley SM, Goldberg DE. 1999. A set of independent selectable markers for transfection of the human malaria parasite *Plasmodium falciparum*. Proc Natl Acad Sci USA 96(15):8716-8720.

van der Wel AM, Tomas AM, Kocken CH, Malhotra P, Janse CJ, Waters AP, Thomas AW. 1997. Transfection of the primate malaria parasite *Plasmodium knowlesi* using entirely heterologous constructs. J Exp Med 185(8):1499-1503.

van Dijk MR, Janse CJ, Waters AP. 1996. Expression of a *Plasmodium* gene introduced into subtelomeric regions of *Plasmodium berghei* chromosomes. Science 271(5249):662-665.

van Dijk MR, Vinkenoog R, Ramesar J, Vervenne RA, Waters AP, Janse C. 1997. Replication, expression and segregation of plasmid-borne DNA in genetically transformed malaria parasites. Mol Biochem Parasitol 86(2):155-162.

van Dijk MR, Waters AP, Janse CJ. 1995. Stable transfection of malaria parasite blood stages. Science 268(5215):1358-1362.

VanWye JD, Haldar K. 1997. Expression of green fluorescent protein in *Plasmodium falciparum*. Mol Biochem Parasitol 87(2):225-229.

Wu, Y, Kirkman LA, Wellems TE. 1996. Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proc Natl Acad Sci USA 93(3):1130-1134.

Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE. 1995. Transfection of *Plasmodium falciparum* within human red blood cells. Proc Natl Acad Sci USA 92(4):973-977.

X. Alternative protocol using DNA-loaded red blood cells for transfection

by Kirk Deitsch

Microbiology and Immunology, Joan and Sanford I. Weill Graduate School of Medical Sciences of Cornell University, New York, NY 10021, USA

e-mail: kwd2001@med.cornell.edu

Equipment

Electroporator (Bio-Rad)

Materials and reagents

Incomplete Cytomix (see Wu et al. 1995):

8.95 g KCl

0.017 g CaCl_2

0.76 g EGTA

1.02 g MgCl_2

0.871 g K_2HPO_4

0.68 g KH_2PO_4

7.08 g HEPES

Dissolve ingredients in water, adjust pH to 7.6 by adding NaOH, and adjust final volume to 1 liter. Filter-sterilize and use.

Preparation

The procedure outlined below is a modification of the above protocol and takes advantage of the observation that malaria parasites actively take up DNA from the cytoplasm of DNA-loaded red blood cells.

- Using a stock of red blood cells stored at 50% hematocrit, transfer 350 μL of cells to a 10- or 15-mL centrifuge tube and spin them down. Remove the supernatant and wash the pellet with 5 mL of Incomplete Cytomix. Spin down the cells again and remove the supernatant.
- Resuspend the cells in Cytomix containing 50 to 100 μg of plasmid DNA. The final volume should be 400 μL . Transfer the cells to a 0.2-cm cuvette and place it on ice.
- Electroporate the cells using the conditions outlined above. We are using the following conditions: voltage: 0.31 kV; capacitance: 960 μFD ; time constants should be in the 10 to 14 ms range.
- Rinse the cells from the cuvette with 5 mL of culture medium. Two consecutive washes of 2.5 mL each works well. Transfer the cells to a centrifuge tube.
- Spin down DNA-loaded red blood cells and remove the supernatant. The supernatant should be quite red from cell lysis, however if nearly all of the red cells have lysed, check the pH of the Cytomix. It should be ~ 7.6 .
- Transfer the cells to a culture flask and inoculate with parasites. Typically the red cells from two electroporations are used for a 5-mL culture. Parasites will take up and express DNA as they invade the DNA-loaded red blood cells.

Advantages of using DNA-loaded red blood cells

- Flasks containing DNA-loaded red blood cells can be inoculated with 100 to 200 μL of an established culture. Such parasites tend to be healthier because they have not been electroporated and give higher levels of transient expression compared to the same starting numbers of parasites transfected directly by electroporation.

- High levels of expression can be obtained by first loading red cells as described above, then inoculating the cells with mature stage parasites purified by Percoll/sorbitol gradients as outlined in PARASITES, section IV:D. As the cells infected by the mature stages rupture, the resulting merozoites invade the DNA-loaded red blood cells resulting in very high numbers of synchronous, transfected parasites.
- If extremely high levels of transient expression are desired, a culture of parasites can be continuously maintained with DNA-loaded red blood cells. After four generations (8 days) of parasite multiplication in DNA-loaded cells, maximal levels of expression are observed. These levels can be up to 20-fold higher than that observed after direct electroporation of parasite-infected cells.

References

Deitsch K, Driskill C, Wellems T. 2001. Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes. *Nucleic Acids Res* 29(3):850-853.

Deitsch KW, Calderwood MS, Wellems TE. 2001 Cooperative silencing elements in var genes. *Nature* 412(6850):875-876.

Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE. 1995. Transfection of *Plasmodium falciparum* within human red blood cells. *Proc Natl Acad Sci USA* 92(4):973-977.

XI. Selectable episomal transfection of asexual stage *Plasmodium falciparum* parasites

by **Amar Bir Singh Sidhu** and **David A. Fidock**

Department of Microbiology and Immunology, Albert Einstein College of Medicine,
Forchheimer 403, 1300 Morris Park Avenue, The Bronx, New York, NY 10461, USA
e-mail: asidhu@aecom.yu.edu, dfidock@aecom.yu.edu

Equipment

Gene Pulser electroporator with Capacitance Extender (Bio-Rad)
electroporation cuvettes (0.2-cm gap, Bio-Rad)
centrifuge
incubator (37 °C)
microscope with 100× lens
modular incubator chambers (Billups–Rothenberg)

Materials and reagents for plasmid preparation

electrocompetent or chemically competent *Escherichia coli* (We prefer XL10-Gold or DH5α for propagation of plasmids containing *P. falciparum* DNA.)
TE buffer:
1 mM EDTA
10 mM Tris–HCl (pH 7.6)
incomplete Cytomix buffer:
120 mM KCl
0.15 mM CaCl₂
2 mM EGTA
5 mM MgCl₂
10 mM K₂HPO₄/KH₂PO₄
25 mM HEPES
Plasmid Maxiprep columns (Qiagen)
Amicon Centricon 100 columns (Millipore)

Materials and reagents for *P. falciparum* propagation

P. falciparum strains
gas mixture of 5% O₂, 5% CO₂, and 90% N₂
human red blood cells (RBC) washed free of leukocytes
RPMI 1640 with L-glutamine (Invitrogen, Catalog No. 31800)
hypoxanthine (Sigma)
HEPES (Sigma)
sodium bicarbonate (Invitrogen)
gentamycin (Invitrogen)
Albumax (I or II, Invitrogen)
complete medium
RPMI 1640 with L-glutamine
50 mg/liter hypoxanthine
10 mg/liter gentamycin
25 mM HEPES
0.225% NaHCO₃
0.5% Albumax
Parafilm

sterile 6-well tissue culture plates
sterile glass pipettes and other tissue culture plastic ware
WR99210 (available upon request from David Jacobus, Jacobus
Pharmaceuticals, Princeton, NJ, USA, e-mail: dpjacobus@aol.com)
blasticidin S HCl (Sigma)
pyrimethamine (Sigma)

This protocol describes fundamental steps for selecting episomally transfected asexual-stage *P. falciparum* parasite lines:

- A. Preparation of parasite cultures and DNA constructs
- B. Electroporation of circular plasmid DNA into parasites
- C. Maintenance of transfected parasite lines

XI:A. Preparation of parasite cultures and DNA constructs

Parasite cultures

For each transfection, use 1×10^7 to 4×10^7 ring stage parasitized RBCs (PRBCs). When preparing parasite cultures for transfection, decrease the hematocrit of the culture from the normal 4% to between 2.5 and 3.0%. Reducing the hematocrit and providing regular medium changes will help obtain higher percentages of ring stage parasites in culture. Because washed human RBCs deteriorate after 4 weeks at 4 °C, infuse the parasite cultures with fresh RBCs shortly before transfection. Feed the parasite cultures regularly to provide nutrients and to prevent buildup of toxic wastes.

The day before transfection, adjust the parasite cultures to between 1.8 and 2.5% parasitemia, rich in trophozoite stages, with addition of fresh RBCs. This parasitemia usually gives 4 to 7% ring stage cultures the next day. Feed the parasite cultures in the late evening again to get healthy ring stage parasites the next day. On the day of transfection, smear and feed the parasites early in the morning and allow them to grow for about 3 to 4 h prior to electroporation. Check that the parasite cultures provide healthy rings and RBCs for transfection and determine the parasitemia (best is about 4 to 6%).

DNA constructs

Prepare pure supercoiled plasmid DNA using Maxiprep columns. Dissolve the DNA in TE buffer at a concentration of 1 to 2 mg/mL. Before transfection, exchange the TE buffer with incomplete Cytomix buffer using Amicon Centricon 100 columns (see below). Store the plasmid DNA equilibrated with incomplete Cytomix buffer at 4 °C, so that whenever parasites are ready, transfection can be performed. Typically, use 50 to 100 µg of plasmid DNA per transfection.

- To exchange TE buffer with incomplete Cytomix buffer for transfection, load approximately 300 µg of plasmid DNA onto an Amicon 100 column.
- Make the volume up to 2.0 mL with filter-sterilized incomplete Cytomix buffer. Cover the top of the column with Parafilm and puncture the Parafilm several times with a sterile 21-23 gauge needle.
- Spin the loaded column in an Amicon collection tube at 3,000 rpm at 4 °C. It usually takes about 40 to 60 min to concentrate.
- After the spin, add 100 to 200 µL of incomplete Cytomix buffer to the column membrane and gently pipette up and down with the residual incomplete Cytomix

buffer in the column, avoiding rupture of the column membrane. Remove the column from the collection tube, flip it upside down into a sterile Eppendorf tube and spin at 3,000 rpm for 5 min to recover the plasmid DNA.

- Measure the volume of eluted plasmid DNA and determine the volume required to transfect the parasites with 100 µg of plasmid DNA.

The design of the construct, the choice of selectable marker, and the selection of the initial transformation plasmid all depend upon the transfection strategy. Plasmids are most often designed to achieve gene disruption ("knockout") or allelic replacement (e.g., to exchange polymorphic codons). Selectable markers established for stable episomal transfection of *P. falciparum* include *Toxoplasma gondii* dihydrofolate reductase–thymidylate synthase (*dhfr-ts*), human dihydrofolate reductase (*dhfr*, with or without the gene encoding green fluorescent protein as a fusion product), blasticidin S deaminase (from *Aspergillus terreus*), neomycin phosphotransferase II (transposon Tn5), and puromycin-*N*-acetyltransferase (from *Streptomyces alboniger*). These markers confer resistance to pyrimethamine, WR99210, blasticidin S HCl, geneticin/G418, and puromycin respectively. When using *T. gondii dhfr-ts*, be careful to transfect pyrimethamine-sensitive parasites. The other selectable markers listed above can be used against antimalarial drug-resistant and drug-sensitive parasite strains.

Transfection plasmids are available for both allelic exchange and transgene expression strategies. One recent addition is the development of constructs expressing thymidine kinase as a negative selectable marker. The use of this marker, in conjunction with the positive marker human *dhfr*, reportedly enables the selection of parasites in which the plasmids have integrated via double crossover events. This recent approach will help in selecting for recombination into the desired locus (as compared to recombination into genomic sequences that have homology to noncoding, regulatory elements present elsewhere on the transfection plasmids). Further, this approach should help in selecting for integration events that confer a deleterious growth phenotype relative to episomally transfected or wild-type parasites.

While not covered by this protocol, techniques and plasmids also exist for transient transfection assays in *P. falciparum*, for example to study regulatory elements. These plasmids typically use luciferase or chloroamphenicol acetyl-transferase (CAT) for signal detection.

XI:B. Electroporation of circular plasmid DNA into parasites

Before starting the transfection experiment, label all electroporation cuvettes and tissue culture plates for parasite culture. Set the Bio-Rad Gene pulser to 0.31 kV, 950 µF, and infinity resistance. Prepare a stock of RBC at 4% hematocrit in complete medium. The whole transfection procedure should be finished in about 30 to 60 min from the time the parasites are harvested to the time the transfected parasites are put back into the incubator.

We note that the procedure listed below relies on transfection of parasitized RBC. An alternative approach recently published by Kirk Deitsch and colleagues involves transfecting *P. falciparum* by first electroporating uninfected RBC with plasmid DNA, then adding mature parasite stages and allowing them to invade the plasmid DNA-preloaded RBC. This is an effective means of obtaining relatively high levels of

luciferase activity and may well represent an improvement for selectable episomal transfection. Nonetheless, the protocol listed below is reliable and works well.

- For each transfection, harvest 2.5 to 3.0 mL of predominantly ring stage parasite culture into a sterile centrifuge tube and spin down the culture at 2,200 rpm for 4 min at room temperature.
- Aspirate the supernatant using a sterile glass pipette.
- Gently resuspend the parasite culture pellet in 3 mL of sterile incomplete Cytomix buffer and spin the culture again at 2,200 rpm for 2 min at room temperature. Aspirate the supernatant as described above.
- After washing the parasite culture with incomplete Cytomix buffer, resuspend the parasite pellet in the required volume of incomplete Cytomix buffer containing plasmid DNA. Measure the volume of this parasite culture–plasmid DNA suspension and make the final volume up to 400 μ L with incomplete Cytomix buffer.
- Transfer the suspension into a Bio-Rad Gene Pulser cuvette (0.2-cm), avoiding any air bubbles.
- Perform the electroporation (using the 0.31 kV, 950 μ F conditions described above). Time constants between 7 and 11 milliseconds give good parasite transfection efficiency.
- Immediately after the electroporation, aseptically add 1 mL of fresh complete medium into the Gene Pulser cuvette. Add the medium gently down the side of the cuvette as the RBC are fragile at this point.
- When all the electroporations are completed, transfer the Gene Pulser cuvettes back to the tissue culture hood. Using a 1-mL pipette, gently remove the cuvette contents into 6-well tissue culture plates (one well per transfection). Pipette 1 mL of complete medium into the cuvette and gently remove most of culture, leaving behind the lysed and dried culture that accumulates around the top of the cuvette.
- Add 3.0 mL of the pre-prepared RBC/complete medium mixture (4% hematocrit) into the well containing the 2 mL of culture from the electroporated material. Mix gently with a 5-mL pipette.
- When all the electroporations are finished, place the 6-well tissue culture plate into a modular chamber, gas the chamber with 5% O₂, 5% CO₂, and 90% N₂, and place it back into a 37 °C tissue culture incubator.
- Three to four hours after electroporation, remove the cultures from the wells and spin them at 2,000 rpm for 4 min at room temperature. Discard the supernatants.
- Resuspend each parasite pellet in 5 mL of fresh complete medium, put it back into the appropriate well of the tissue culture plate, gas, and return it to the incubator.
- The following day, feed each well with 5 mL of complete medium.

XI:C. Maintenance of transfected parasite lines

- On the second day post-transfection, smear the electroporated parasite cultures to determine their parasitemia. A parasitemia of 0.8 to 2.4% at this time point is usually indicative of relatively good transfection efficiency. For most plasmids, this typically means that episomally transfected parasite lines will be first observed in 2 to 3 weeks. From this day onward, feed parasites with medium

containing the selectable agent. To select for human *dhfr*, blasticidin S-deaminase, or *T. gondii dhfr-ts*, we recommend using complete medium supplemented with 2.5 nM WR99210, 2.5 µg/mL of blasticidin S HCl, or 20 ng/mL of pyrimethamine respectively.

- Feed the parasites daily on *Days 3-5*.
- On *Day 6*, smear the lines. There should be no live asexual stage parasites. Large numbers of gametocytes are a sign of excessive stress during the transfection and/or subsequent handling procedures. Reasons for this can include poor parasite health prior to transfection, too much time spent outside of the incubator, or poor RBC condition. Similarly, it is not good to see too many “blebby” RBC. (If needed, these RBC can be diluted out by replacing 20 to 30% of the culture with fresh uninfected RBC at this time.)
- Resuspend the *Day 6* cultures in 5 mL of complete medium containing the drug and transfer them into a sterile centrifuge tube. Spin the tube at 1,800 rpm for 3 min at room temperature; this centrifugation helps to remove toxic lysis products and dead parasite material. Aspirate and discard the supernatant. Resuspend the pellet in 5 mL of medium containing both drug and an extra 0.1 mL of RBC (stock of 50% hematocrit).
- Skip *Day 7*.
- Feed again on *Day 8*.
- Skip *Day 9*.
- Feed again on *Day 10*. Repeat the smearing, centrifugation, and addition of fresh RBC procedures performed on *Day 6*. There should be minimal parasite debris left with few gametocytes and the RBC should be in good condition. Note: From *Day 10* onwards, it is possible to reduce culture volumes from 5 mL down to 1 mL per line until parasites are observed, then the lines can be expanded back out to 5 mL again for freezing.
- Feed every second day (*Days 12, 14* etc.) and smear every 4 days until parasites become microscopically detectable on Giemsa-stained slides. Typically, parasites are first visualized on *Days 14 to 24*. To prevent the parasite cultures from lysing, discard 30 to 40% of the cultures and replace them with fresh RBC every 6 or 7 days.
- Once parasites have been seen, begin feeding them daily. Freeze the transfected parasite lines as soon as the parasitemia reaches $\geq 2\%$ with $>50\%$ rings. Note the growth rate of the episomally transfected cultures, which grow slower than the parental nontransfected lines.
- Expand and harvest the remaining cultures for genomic DNA extraction from trophozoite mature stage parasites to confirm transfection by plasmid rescue. Subsequently, parasite lines can be maintained in 1-mL volumes for long-term propagation.
- Make frozen parasite stocks every month. It is also convenient to prepare genomic DNA once a month to screen for integration by PCR.
- Stable integrants resulting from homologous recombination and single-site crossover of the plasmid into the genome can often be observed between 45 and 90 days post-transfection. These stable integrant parasites typically grow faster than episomally transfected lines. PCR with primers specific to the vector and the plasmid can be used in combination to efficiently screen for the presence in the bulk culture of parasites that underwent the desired recombination event. PCR positive data can be confirmed by Southern hybridization. Once the desired

integration has been detected, the corresponding parasite lines can be cloned by limiting dilution and detected using the Malstat lactate dehydrogenase reagent.

References

- Crabb BS, Cooke BM, Reeder JC, Waller RF, Caruana SR, Davern KM, Wickham ME, Brown GV, Coppel RL, Cowman AF. 1997. Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. *Cell* 89(2):287-296.
- Crabb BS, Cowman AF. 1996. Characterization of promoters and stable transfection by homologous and nonhomologous recombination in *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 93(14):7289-7294.
- Crabb BS, Triglia T, Waterkeyn JG, Cowman AF. 1997. Stable transgene expression in *Plasmodium falciparum*. *Mol Biochem Parasitol* 90(1):131-144.
- Deitsch K, Driskill C, Wellems T. 2001. Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes. *Nucleic Acids Res* 29(3):850-853.
- Deitsch KW, Calderwood MS, Wellems TE. 2001. Malaria: cooperative silencing elements in *var* genes. *Nature* 412(6850):875-876.
- de Koning-Ward TF, Waters AP, Crabb BS. 2001. Puromycin-N-acetyltransferase as a selectable marker for use in *Plasmodium falciparum*. *Mol Biochem Parasitol* 117(2):155-160.
- Duraisingh MT, Triglia T, Cowman AF. 2002. Negative selection of *Plasmodium falciparum* reveals targeted gene deletion by double crossover recombination. *Int J Parasitol* 32(1):81-89.
- Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu ABS, Naude B, Deitsch K, et al. 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell* 6(4):861-871.
- Fidock DA, Nomura T, Wellems TE. 1998. Cycloguanil and its parent compound proguanil demonstrate distinct activities against *Plasmodium falciparum* malaria parasites transformed with human dihydrofolate reductase. *Mol Pharmacol* 54(6):1140-1147.
- Fidock DA, Wellems TE. 1997. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. *Proc Natl Acad Sci USA* 94(20):10931-10936.
- Goodyer ID, Taraschi TF. 1997. *Plasmodium falciparum*: a simple, rapid method for detecting parasite clones in microtiter plates. *Exp Parasitol* 86(2):158-160.

- Kadekoppala M, Kline K, Akompong T, Haldar K. 2000. Stable expression of a new chimeric fluorescent reporter in the human malaria parasite *Plasmodium falciparum*. *Infect Immun* 68(4):2328-2332.
- Mamoun CB, Gluzman IY, Goyard S, Beverley SM, Goldberg DE. 1999. A set of independent selectable markers for transfection of the human malaria parasite *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 96(15):8716-8720.
- Templeton TJ, Kaslow DC, Fidock DA. 2000. Developmental arrest of the human malaria parasite *Plasmodium falciparum* within the mosquito midgut via CTRP gene disruption. *Mol Microbiol* 36(1):1-9.
- Triglia T, Wang P, Sims PF, Hyde JE, Cowman AF. 1998. Allelic exchange at the endogenous genomic locus in *Plasmodium falciparum* proves the role of dihydropteroate synthase in sulfadoxine-resistant malaria. *EMBO J* 17(14):3807-3815.
- Waller RF, Keeling PJ, Donald RG, Striepen B, Handman E, Lang-Unnasch N, Cowman AF, Besra GS, Roos DS, McFadden GI. 1998. Nuclear-encoded proteins target to the plastid in *Toxoplasma gondii* and *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 95(21):12352-12357.
- Wu, Y, Kirkman LA, Wellems TE. 1996. Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. *Proc Natl Acad Sci USA* 93(3):1130-1134.
- Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE. 1995. Transfection of *Plasmodium falciparum* within human red blood cells. *Proc Natl Acad Sci USA* 92(4):973-977.

XII. Telomere repeat amplification protocol in *Plasmodium falciparum* (PfTRAP)

by **Nasséra Bakhsis¹**, **Emmanuel Bottius¹**, **Luísa M. Figueiredo²**, and **Artur Scherf¹**

¹Institut Pasteur, Unité BIHP, 25 rue du Dr. Roux, 75015 Paris, France

e-mail: ascherf@pasteur.fr

²Laboratory of Molecular Parasitology, Rockefeller University, 1230 York Avenue, New York, NY 10021, USA

e-mail: Luisa.Figueiredo@mail.rockefeller.edu

Equipment

homogenizer (Kontes; size 19)
slides and coverslips
optical microscope
centrifuges (15-mL tube and microfuge)
PCR machine
vertical electrophoresis system (10 × 8 cm)

Materials and reagents

parasite culture of 10% parasitemia containing ~10⁹ parasites
(mostly 16- to 30-h cultures)

Buffer B:

10 mM Tris–HCl (pH 7.5)
1 mM MgCl₂
1mM EGTA
5 mM 2-mercaptoethanol
10% glycerol
10 µg/mL leupeptin
10 µg/mL pepstatin

TRAP buffer:

200 mM Tris–HCl (pH 8.3)
15 mM MgCl₂
630 mM KCl
0.05% Tween 20
10 mM EGTA
500 µM dNTP
1 mg/mL BSA

Oligonucleotides:

PfTS (5' AATCCGTCGAGCAGAGTTCA 3')
PfCX (5' GGCGCGT G/A AACCCCT G/A AACCCCT G/A AACCC 3')
a GC-clamp followed by 3 telomere repeats)

Preparation

- Wash the homogenizer carefully and rinse it with 0.4 M NaOH, 0.25 M HCl, 0.4 M NaOH, and distilled water successively. Put it on ice before use.
- Use only RNase-free solutions and materials for the TRAP assay.
- Wear gloves all the time and keep parasite extracts on ice.

Preparation of a semipurified protein extract (~3 h)

- Do a saponin lysis of a freshly recovered culture (described elsewhere in this book) containing $\sim 10^9$ parasites.
- Wash the pellet in 5 volumes of PBS to eliminate the majority of erythrocyte contaminants.
- Wash the pellet in Buffer B.
- Resuspend the parasite pellet in 200 μL of Buffer B.
- Transfer parasites to the homogenizer and lyse the parasitophorous vacuole and cytoplasmic membrane by applying 80 strokes on ice.
- Carefully, take $\sim 2 \mu\text{L}$ of the lysate and put it between the slide and a coverslip.
- Using a phase contrast optical microscope, check that the parasitophorous vacuole and cytoplasmic membrane have been disrupted (resulting in debris in the suspension). If not, apply 80 more strokes. Repeat this procedure until more than 90% of the parasites have been lysed.
- Transfer the lysate to a prechilled 1.5-mL tube.
- Centrifuge the lysate for 1 hr at $17,600 \times g$ at 4°C .
- The supernatant consists of a semipurified cytoplasmic fraction at a concentration of 5×10^6 parasite equivalents per microliter. Aliquot it in 2-, 4-, and 12- μL amounts and store it at -80°C .
- The pellet containing the nuclei can be resuspended in 200 μL of Buffer B, lysed by sonication, and centrifuged as described above.
- Both cytoplasmic and nuclear fractions contain telomerase activity. For practical reasons we usually use the cytoplasmic fraction.

TRAP assay (~6 h)

The TRAP assay consists of an elongation and an amplification step. In the elongation step, an oligonucleotide (PfTS) that mimics a chromosome extremity is used as a substrate for telomerase. The amplification step consists of a PCR in which the products from the elongation step are amplified and labelled with ^{32}P .

Elongation step:

- Use enough DNA in the reaction (equivalent to about 10^7 parasites) to get a positive reaction.
- In an RNase-free PCR tube, prepare the following mix:
 - 2 μL of protein extract
 - 5 μL of TRAP buffer
 - 100 ng of PfTS oligonucleotide
 - H_2O to 48.5 μL
- Incubate the mix at 37°C for 1 h.

Amplification step:

- To each tube containing the elongation products add:
 - 100 ng of PfCX oligonucleotide
 - 2.5 μCi ^{32}P -dNTP
 - 2.5 U Ampli *Taq* Gold DNA polymerase (Applied Biosystems)
 - H_2O up to final volume of 50 μL
- Amplify as follows:
 - 1 cycle: 95°C for 10 min
 - 35 cycles: 95°C for 10 s, 55°C for 30 s, 72°C for 1 min

- Run 2 controls for nontelomerase-mediated incorporation. Before starting the elongation step, (i) pretreat semipurified protein extract for 30 min at 37 °C with 10 µg of RNase A and (ii) denature it at 95 °C for 10 min.
- Resolve PCR products on a 15% nondenaturing 8 × 10-cm polyacrylamide gel, at 150V (~2 h), in 1× TBE.
- At the end of electrophoresis, disassemble apparatus, leaving the gel stuck to one of the glass plates. Cover it with Saran wrap and expose it (without drying) to Kodak film for a few hours.

Reference

Bottius E, Bakhsis N, Scherf A. 1998. *Plasmodium falciparum* telomerase: de novo telomere addition to telomeric and nontelomeric sequences and role in chromosome healing. Mol Cell Biol 18(2):919-925.

XIII. Genotyping of *Plasmodium falciparum* parasites by PCR: *msp1*, *msp2*, and *glurp*

by **Georges Snounou¹** and **Anna Färnert²**

¹Unité de Parasitologie Biomédicale et CNRS URA 1960, Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France

e-mail: snounou@pasteur.fr

²Division of Infectious Diseases, Department of Medicine, Karolinska Institutet, Karolinska Hospital, SE-171 76 Stockholm, Sweden

e-mail: anna.farnert@medks.ki.se

Equipment

micropipettes and tips
thermal cycler
apparatus for agarose gel electrophoresis
photographic equipment and UV transilluminator
refrigerator (4 °C) and freezer (−20 °C) for reagent storage
microcentrifuge

Materials and reagents

Taq polymerase (store at −20 °C) with appropriate buffer (store at 4 °C)
MgCl₂ stock solution (often provided by enzyme supplier)
dNTP: a working solution with a concentration of 5 mM for each of dATP, dCTP, dGTP and dTTP (store at −20 °C)
oligonucleotide primers (sequences are given below): a working solution with a concentration of 2.5 µM for each oligonucleotide primer (store at −20 °C)
mineral oil
loading buffer (5×):
 50 mM Tris (pH 8.0)
 75 mM EDTA (pH 8.0)
 0.5% SDS
 30% w/v sucrose
 10% Ficoll (w/v, average molecular weight 400 000)
 0.25% orange G dye (w/v approximately)
10× TBE buffer:
 1 M Tris
 1 M boric acid
 50 mM EDTA
 A pH of approximately 8.3 should be obtained without any adjustment.
Agaroses:
 normal agarose
 high resolution agaroses (such as NuSieve or MetaPhor, Cambrex)
ethidium bromide solution containing 10 mg per mL of water
 CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood. Store in the dark at 4 °C.
pure water

Genotyping of *P. falciparum* infections permits characterization of distinct subpopulations present in an isolate. The genetic markers merozoite surface protein (*msp1*) (block2),

msp2, and glutamate rich protein (*glurp*) have been chosen due to their extensive polymorphism in size and sequences. The different allelic types, i.e., families, identified for *msp1* (K1, MAD 20, and RO33 types) and *msp2* (FC27 and IC/3D7) are detected with specific primers in a second nested PCR.

The amplification strategy used for genotyping *P. falciparum* parasites is the nested PCR. In the first amplification reaction oligonucleotide primer pairs, which will hybridize to conserved sequences flanking the repeat polymorphic regions of the genes, are used. The product of this first reaction is then used as a DNA template for separate second amplification reactions in which the oligonucleotide primers used recognise sequences contained within the DNA fragment amplified in the first reaction.

Setting up the first amplification reaction

- Calculate the total volume of reaction mixture required: 20 µL per reaction × (total number of reactions + 1).
- Calculate the amount of:
 - buffer (final concentration 1×)
 - MgCl₂ (final concentration 1.5 mM)
 - dNTP (final concentration 125 µM)
 - oligonucleotides (final concentration 250 nM)
 - Taq* polymerase (final concentration 2 units per 100 µL)
 - and water to make up the total volume

NB: If all three genetic markers (*msp1*, *msp2*, and *glurp*) are to be analysed, then a mixture of the corresponding 3 oligonucleotide primer pairs can be added to the reaction. Thus one first amplification reaction only is required for each sample.

- Add, in order, the correct volumes of: water, buffer, MgCl₂, oligonucleotide primers, dNTP, and the *Taq* polymerase. Mix by a short vortex pulse.
- Aliquot 20 µL of the reaction mixture per labelled tube.
- Add 40 µL of mineral oil overlay to each tube.
- Add 1 µL of DNA template to each tube (water for the negative controls).
- Place in the cyclor and run the PCR reaction.

Setting up the second amplification reactions

Steps 1 – 5 as above.

- Remove 1 µL from the first amplification reaction tube from under the oil overlay and add it to the oil overlay of the second amplification reaction tube.
- Place the tube in the cyclor and run the PCR reaction.

NB: Each second amplification reaction must be performed with a single oligonucleotide pair. Thus, per sample, 3 separate reactions will be required for the analysis of *msp1*, two for *msp2*, and one for *glurp*.

Cycling parameters

Step 1	95 °C for 5 min	Initial denaturation
Step 2	X °C for 2 min	Annealing
Step 3	72 °C for 2 min	Extension
Step 4	94 °C for 1 min	Denaturation
Step 5	Repeat Steps 2–4 a total of 25 cycles (Nest 1) or 30 cycles (Nest 2)	
Step 6	X °C for 2 min	Final annealing
Step 7	72 °C for 5 min	Final extension
Step 8	The reaction is completed by reducing the temperature to 25 °C.	

X = 58 °C for Nest 1 (M1-OF/M1-OR; M2-OF/M2-OR; G-OF/G-OR) and the Nest 2 reactions of *glurp* (G-NF/G-OR).

X = 61 °C for Nest 2 reactions of *msp1* (M1-KF/M1-KR; M1-MF/M1-MR; M1-RF/M1-RR) and of *msp2* (M2-FCF/M2-FCR; M2-ICF/M2-ICR).

Sensitivity and specificity

- The parameters provided above (cycling parameters and final concentrations) may have to be altered in order to obtain optimal sensitivity and specificity. The use of different temperature cyclers and enzymes has an influence on the efficiency of the PCR reaction. The nested PCR for the 3 genetic markers should be capable of detecting about 10 parasite genomes per microliter of blood.
- Optimization of the PCR conditions requires the preparation of a standard set of genomic DNA templates. These are prepared from a defined quantity of in vitro cultured cloned parasite lines. Cloned lines must be used since the haploid genome contains a single copy of each of the 3 genes. If needed the conditions are varied in such a way that a single band is obtained for each of the specific second amplification reactions for a range of parasite genomic DNA concentrations.
- Sensitivity is mainly dependent on the number of cycles, thus these can be increased if needed.
- Specificity is most affected by the annealing temperature and eventually the MgCl₂ concentration. A common problem is the generation of two PCR products for the specific second amplification reactions. This is due to carryover of oligonucleotide primers and PCR product from the first reaction, which can be minimized by reducing the oligonucleotide concentrations and/or the number of cycles in the first amplification reaction.

Minimizing contamination

- The risks of contamination are enormously increased when nested PCR is performed. Thus the transfer of the product of the first amplification reaction to the second amplification reaction mixture should be performed with extreme care. In this context the oil that is used to overlay the reaction mixtures acts as a very efficient contamination barrier, and it is strongly suggested to retain the practice even when a heated lid is available.
- Ideally, setting up the first and second amplification reactions should be performed in a separate room from the one where the gels are migrated. Moreover, the transfer of the template from the first to the second amplification reactions must be performed with a dedicated pipette in yet another room, preferably with filter tips.

Analysis of the PCR product

- Add 5 µL of the loading buffer to the PCR product.
- Load 12 µL of sample on a suitable agarose gel and migrate (1× TBE buffer).
- Stain the gel in TBE buffer containing ethidium bromide (final concentration 1 µg per mL) for 30 min.
- Destain in TBE or water for 5 min.
- Visualise on a UV transilluminator.

Normal agarose is suitable for the analysis of all 3 genetic markers. However, given the small size of the bands which will result from the amplification of *msp1*, and the small variations in the sizes of the different allelic variants, the use of agarose type which give higher resolution is advised. The best results are obtained if the gel is kept cold before and during electrophoresis. The high cost of such agarose types (NuSieve or MetaPhor) can be compensated by the fact that the gels can be reused at least 10 times without significant loss of resolution. Used gels can be stored in TBE buffer, and when needed for further use, they are just reboiled. To compensate for condensation, add up water to the original gel volume when reboiling.

References

Arez AP, Lopes D, Pinto J, Franco AS, Snounou G, do Rosario VE. 2000. *Plasmodium* sp.: optimal protocols for PCR detection of low parasite numbers from mosquito (*Anopheles* sp.) samples. Exp Parasitol 94(4):269-272.

Färnert A, Arez AP, Babiker HA, Beck HP, Benito A, Björkman A, Bruce MC, Conway DJ, Day KP, Henning L, et al. 2001. Genotyping of *Plasmodium falciparum* infections by PCR: a comparative multicentre study. Trans R Soc Trop Med Hyg 95(2):225-232.

Färnert A, Arez AP, Correia AT, Björkman A, Snounou G, do Rosario V. 1999. Sampling and storage of blood and the detection of malaria parasites by polymerase chain reaction. Trans R Soc Trop Med Hyg 93(1):50-53.

Snounou G. 2002. Genotyping of *Plasmodium* spp. Nested PCR. Methods Mol Med 72:103-116. [This reference includes a detailed account of the protocol.]

Snounou G, Beck HP. 1998. The use of PCR-genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment. Parasitol Today 14(11):462-467.

Snounou G, Zhu X, Siripoon N, Jarra W, Thaitong S, Brown KN, Viriyakosol S. 1999. Biased distribution of *msp1* and *msp2* allelic variants in *Plasmodium falciparum* populations in Thailand. Trans R Soc Trop Med Hyg 93(4):369-374. (NB: The order of the *msp1* K1 and MAD20 oligonucleotide was inverted in Table 1).

Oligonucleotide sequences

Merozoite Surface Protein 1: *msp1*

First reaction: M1-OF 5'- CTAGAAGCTTTAGAAGATGCAGTATTG -3'
M1-OR 5'- CTAAATAGTATTCTAATTCAAGTGGATCA -3'

Second reaction:

K1-type M1-KF 5'- AAATGAAGAAGAAATTACTACAAAAGGTGC -3'
M1-KR 5'- GCTTGATCAGCTGGAGGGCTTGACCCAGA -3'
MAD20-type M1-MF 5'- AAATGAAGGAACAAGTGAACAGCTGTTAC -3'
M1-MR 5'- ATCTGAAGGATTTGTACGTCTTGAATTACC -3'
RO33-type M1-RF 5'- TAAAGGATGGAGCAAATACTCAAGTTGTTG -3'
M1-RR 5'- CATCTGAAGGATTTGCAGCACCTGGAGATC -3'

Merozoite Surface Protein 2: *msp2*

First reaction: M2-OF 5'- ATGAAGGTAATTAAAACATTGTCTATTATA -3'
M2-OR 5'- CTTTGTTACCATCGGTACATTCTT -3'

Second reaction:

FC27-type M2-FCF 5'- AATACTAAGAGTGTAGGTGCGATGCTCCA -3'
M2-FCR 5'- TTTTATTTGGTGCATTGCCAGAACTTGAAC -3'
IC/3D7-type M2-ICF 5'- AGAAGTATGGCAGAAAGTAAKCCTYCTACT -3'
M2-ICR 5'- GATTGTAATTCGGGGGATTCAGTTTGTTTCG -3'

Glutamate Rich Protein: *glurp*

First reaction: G-OF 5'- TGAATTTGAAGATGTTCACTGAAC -3'
G-OR 5'- GTGGAATTGCTTTTTCTTCAACTAA -3'

Second reaction:

G-NF 5'- TGTTCACTGAACAATTAGATTTAGATCA -3'
G-OR 5'- GTGGAATTGCTTTTTCTTCAACTAA -3'

FIELD BLOOD SAMPLING

I. Finger prick blood sampling

by **Akira Kaneko**

*Tokyo Women's Medical University, Department of Tropical Medicine, Tokyo 162-8666, Japan
and Malaria Research Laboratory, Department of Medicine, Karolinska Hospital, SE-171 76
Stockholm, Sweden*

e-mail: akirak@research.twmu.ac.jp

I:A. Blood collection on filter paper

Equipment

heating block (Techne)

Materials and reagents

heparinized capillary tubes, 75- μ L (Drummond Scientific)

chromatography filter paper, ET31CHR (Whatman).

EDTA tubes (Microtainer, Becton Dickinson)

Procedure

- Draw finger prick blood samples into 1 to 3 heparinized, 75- μ L capillary tubes and transfer them to chromatography filter paper.
- Alternatively, allow 1 drop of blood to fall onto a filter paper directly from the finger. The finger must not touch the filter paper (see the photo).
- Soak the drop into the filter paper until the paper is evenly red without spreading to the underlying surface.
- Dry the filter-paper samples completely in air (see the photo) and place them in small plastic bags.
- We can keep the dried filter-paper samples at room temperature for at least several weeks in field conditions before extracting antibodies, human and parasite DNA, and postdose drugs.
- Store the filter-paper samples at -20°C for long term.



I:B. Blood collection in EDTA tubes

by Akira Kaneko

*Tokyo Women's Medical University, Department of Tropical Medicine, Tokyo 162-8666, Japan
and Malaria Research Laboratory, Department of Medicine, Karolinska Hospital, SE-171 76
Stockholm, Sweden*

e-mail: akirak@research.twmu.ac.jp

A better quality and quantity of DNA can be obtained by EDTA tubes than by filter paper.

Materials and reagents

EDTA tubes (Microtainer, Becton Dickinson)

Procedure

- Draw blood samples taken from the finger tip into 2 to 5 heparinized capillary tubes as mentioned above and transfer into an EDTA tube.
- We can keep the EDTA-tube samples at room temperature for at least one week in field conditions before extracting DNA.
- Store the EDTA-tube samples at -80°C for long term.

II. Serum extraction from filter-paper samples

by **Akira Kaneko**

*Tokyo Women's Medical University, Department of Tropical Medicine Tokyo 162-8666, Japan and
Malaria Research Laboratory, Department of Medicine, Karolinska Hospital, SE-171 76*

Stockholm, Sweden

e-mail: akirak@research.twmu.ac.jp

Equipment

paper punch
shaker (Vortex)

Materials and reagents

10-mL tubes
PBS containing 0.05% Tween and 0.5% BSA
Pasteur pipettes

Procedure

- Use a paper punch with a diameter of about 6 mm for punching out discs. Be careful to use areas in the middle of the spot where the blood is evenly spread.
- Put the discs in 10-mL tubes.
- Add 500 μ L of PBS containing 0.05% Tween and 0.5% BSA.
- Incubate the discs for 2 h at room temperature on a shaker.
- Vortex them for a few seconds.
- Withdraw the liquid with a Pasteur pipette and aliquot it.
- Store at -20°C until analysis.
- The extract corresponds to a serum dilution of $\sim 1:100$. The dilution factor is however dependent on the quality of the filter paper.

III. DNA extraction

III: A. DNA extraction from filter-paper samples

by **Akira Kaneko**

Tokyo Women's Medical University, Department of Tropical Medicine, Tokyo 162-8666,
Japan and Malaria Research Laboratory, Department of Medicine, Karolinska Hospital,
SE-171 76 Stockholm, Sweden
e-mail: akirak@research.twmu.ac.jp

Microscale isolation of *Plasmodium falciparum* or human DNA from filter-paper samples has been conventionally done by brief boiling (Wooden et al. 1992; Uchida et al. 1995), which is sufficient for obtaining short DNA.

Equipment

centrifuge

Materials and reagents

sterile water containing 15 µL of Chelex-100 (Bio-Rad)
HEPES-buffered saline (HBS) containing 0.5% (w/w) saponin (Merck)
1.5-mL tube
QIAamp DNA Mini Kit (Qiagen)
ethanol
DNA elution buffer AE:
0.5 mM EDTA
10 mM Tris-HCL (pH 9.0)

Procedure

- Cut one quarter or half of the dried filter blot, equivalent to 19 or 38 µL of blood, into 3 × 3-mm pieces.
- Heat the pieces in 205 µL of sterile water containing 15 µL of Chelex-100 at 100 °C for 8 min.
- After cooling them on ice for 2 min, collect the supernatant containing DNA (200 µL) by centrifugation and store them at –20 °C.

However, the DNA samples thus obtained show a limited PCR template activity with little amplification of DNA longer than 1 kb. Considering the extremely high A + T content of *P. falciparum* DNA, boiling may cause fragmentation of DNA and would no longer be a method of choice for DNA isolation when a long PCR product covering an entire gene is required. To solve this problem the following alternative method has been developed (Sakihama et al. 2001)

- Cut one quarter or half of the dried filter blot, equivalent to 19 or 38 µL of blood, into 3 × 3-mm pieces.
- Incubate the pieces in 1 mL of HEPES-buffered saline (HBS) containing 0.5% (w/w) saponin at room temperature for 1.5 h in a 1.5-mL tube.
- Wash them twice with 1 mL of HBS.
- Use a QIAamp DNA Mini Kit to isolate DNA remaining on the filter paper, according to the manufacturer's instructions with the following modifications.
- To avoid shearing DNA, minimize vortexing and substitute by repeated inversions.

- Before the addition of ethanol to the extract, thoroughly remove the filter-paper debris by centrifugation at $9,000 \times g$ for 5 min.
- Reduce the volume of DNA elution buffer AE to 50 μL .
- Prolong the preincubation before DNA elution to 5 min to maximize the elution recovery.
- Store the eluted DNA at 4 °C until PCR amplification.

References

Sakihama N, Mitamura T, Kaneko A, Horii T, Tanabe K. 2001. Long PCR amplification of *Plasmodium falciparum* DNA extracted from filter paper blots. *Exp Parasitol* 97(1):50-54.

Uchida JY, Kasahara T, Bobogare A, Saefafia S, Kere N, Kawabata M, Ohta N, Ishii A. 1995. The prevalence of *falciparum* malaria in the Solomon Islands investigated by a filter paper disk PCR method. *Jpn J Parasitol* 44(2):119-127.

Wooden J, Gould EE, Paull AT, Sibley CH. 1992. *Plasmodium falciparum*: a simple polymerase chain reaction method differentiating strains. *Exp Parasitol*. 75(2):207-212.

III:B. DNA extraction from filter-paper discs previously used for serum extraction**by Manijeh Vafa, Masashi Hayano, and Marita Troye-Blomberg***Immunology Department, The Wenner-Gren Institute, Stockholm University,**SE-106 91 Stockholm, Sweden**e-mail: manijeh@imun.su.se, mhayano@imun.su.se, marita@imun.su.se***Equipment**

vortex
PCR machine
centrifuge

Materials and reagents

Eppendorf tubes
saponin
PBS
Chelex-100
PCR tubes
distilled water

Procedure

- After serum extraction as described in FIELD BLOOD SAMPLING, section II, transfer the discs to new Eppendorf tubes containing 1 mL of 0.5% saponin in PBS
- Incubate the tubes at 4 °C overnight.
- Discard the solution.
- Wash the discs with 1 mL of PBS at 4 °C for 15 to 30 min.
- Discard supernatant.
- Transfer the discs into 0.2-mL PCR tubes containing 50 µL of 20% Chelex-100 in water + 150 µL of distilled water.
- Vortex the tubes for 30 s.
- Incubate the mix at 99 °C for 15 min in the PCR machine.
- Vortex for 30 s.
- Centrifuge the tubes at 10,000 rpm for 2 to 3 min.
- Collect the supernatant (using pipette) in new microtubes.
- Use 5 µL for PCR, or store at –20 °C.

III:C. DNA extraction from EDTA-tube samples

by **Akira Kaneko**

*Tokyo Women's Medical University, Department of Tropical Medicine, Tokyo 162-8666,
Japan and Malaria Research Laboratory, Department of Medicine, Karolinska Hospital, SE-
171 76 Stockholm, Sweden*

e-mail: akirak@research.twmu.ac.jp

Equipment

water bath
Generation Capture Plate kit (Gentra Systems)

Materials and reagents

whole blood frozen in EDTA tube
EDTA tubes

Procedure

- Use 150 to 375 μL of frozen ($-80\text{ }^{\circ}\text{C}$) whole blood collected in an EDTA tube.
- Thaw it quickly in a $37\text{ }^{\circ}\text{C}$ water bath and keep it on ice until use.
- Use Generation Capture Plate kit according to the manufacturer's instructions.

III:D. Fast methanol-based DNA extraction from blood spots in filter paper.**by *Pedro Gill and Sandor Bereczky****Malaria Research Laboratory, Department of Medicine, Karolinska Hospital, SE-171 76 Stockholm, Sweden**e-mail: pedro.gill@ks.se, sandor.bereczky@ks.se***Equipment**

heating block (Techne)
micropipette, 1000- μ L

Materials and reagents

blood spots on filter paper (Whatman 3MM)
new surgical blades
tips
1.5-mL Eppendorf tube
methanol
distilled, sterile water

Procedure

- Cut a blood spot (about 3×5 mm) from the filter paper. (Note: Not all filter papers work when making the blood spot collection. Make sure you are using the right one, usually Whatman 3MM.) Do not touch the blood with the fingers when manipulating the filter paper. Use new surgical blades, one for each sample, to avoid contamination. The cuts should be done on a disposable surface, the best choice being small yellow stickers (note pads).
- Use the blade also for transferring the cut-out to a 1.5-mL Eppendorf tube. The filter papers with the remaining blood spots should be protected (e.g., in separate plastic bags) until a new DNA extraction. After each blood sample, remove 2 or 3 note pad sheets (due to the fact that the blade usually cuts through at least two sheets of the note pad).
- Add 125 μ L of methanol (room temperature) and wait 15 min. Make sure that the whole paper-cut is soaked in the methanol. Keep at room temperature until further processed (at least 15 min).
- Remove the methanol with a 1000- μ L micropipette (using a new tip for every sample).
- Let the paper dry at room temperature for 15 min with the lid open. Close the lid and rotate the tube gently. If the paper moves freely it is dry, if it sticks even slightly, dry it for 5 to 10 more minutes.
- Add 65 to 75 μ L of distilled, sterile water and smash the paper in the water with the micropipette. The water will become slightly red.
- Heat the tube at 95 to 100 °C in a heating block or a water bath for 15 min.
- Ready for PCR! Use 3 to 10 μ L in a 50- μ L total reaction volume. (Attention: This extraction is rather dirty. Too much template can give rise to inhibition of polymerase.)

Note: FIELD BLOOD SAMPLING, section III:A for DNA extraction is suitable for long, intact fragments over 700 bp, and is particularly efficient for fragments more than 1000 bp, while FIELD BLOOD SAMPLING, section III:D is particularly efficient for short fragments up to 500 bp and when the filter paper cut is small (2×2 mm).

References

Evengård B, Linder E, Lundbergh P. 1988. Standardization of a filter-paper technique for blood sampling. *Ann Trop Med Parasitol* 82(3):295-303.

Gil JP, Nogueira F, Strömberg-Nörklit J, Lindberg J, Carrolo M, Casimiro C, Lopes D, Arez AP, Cravo PV, Rosario VE. 2003. Detection of atovaquone and Malarone resistance conferring mutations in *Plasmodium falciparum* cytochrome b gene (cytb). *Mol Cell Probes* 17(2-3):85-89.

Kain KC, Lanar DE. 1991. Determination of genetic variation within *Plasmodium falciparum* by using enzymatically amplified DNA from filter paper disks impregnated with whole blood. *J Clin Microbiol* 29(6):1171-1174.

Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE. 1995. Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. *Am J Trop Med Hyg* 52(6):565-568

Sakihama N, Mitamura T, Kaneko A, Horii T, Tanabe K. 2001. Long PCR amplification of *Plasmodium falciparum* DNA extracted from filter paper blots. *Exp Parasitol* 97(1):50-54.

Uchida JY, Kasahara T, Bobogare A, Saefafia S, Kere N, Kawabata M, Ohta N, Ishii A. 1995. The prevalence of *falciparum* malaria in the Solomon Islands investigated by a filter paper disk PCR method. *Jpn J Parasitol* 44(2):119-127.

Wooden J, Gould EE, Paull AT, Sibley CH. 1992. *Plasmodium falciparum*: a simple polymerase chain reaction method differentiating strains. *Exp Parasitol* 75(2):207-212.

IV. Postdose drug level monitoring

by **Akira Kaneko**

Tokyo Women's Medical University, Department of Tropical Medicine, Tokyo 162-8666, Japan and Malaria Research Laboratory, Department of Medicine, Karolinska Hospital, SE-171 76 Stockholm, Sweden
e-mail: akirak@research.twmu.ac.jp

Using the filter-paper samples, chromatographic analytical methods were developed to monitor capillary blood levels of the drug and its metabolites after administration by Bergqvist and colleagues. Currently methods for the following drugs are available:

References

Bergqvist Y, Churchill FC, Mount DL. 1988. Determination of mefloquine by electron-capture gas chromatography after phosgene derivatisation in biological samples and in capillary blood collected on filter paper. *J Chromatogr* 428(2):281-290.

Bergqvist Y, Funding L, Kaneko A, Krysen B, Leek T. 1998. Improved method for the simultaneous determination of proguanil and its metabolites by high-performance liquid chromatography and solid-phase extraction of 100- μ L capillary blood samples dried on sampling paper. *J Chromatogr B* 719(1-2):141-149.

Bergqvist Y, Hjelm E, Rombo L. 1987. Sulfadoxine assay using capillary blood samples dried on filter paper-suitable for monitoring of blood concentrations in the field. *Ther Drug Monit* 9(2):203-207.

Lindegardh N, Forslund M, Green MD, Kaneko A, Bergqvist Y. 2002. Automated solid-phase extraction for determination of amodiaquine, chloroquine, and metabolites in capillary blood on sampling paper by liquid chromatography. *Chromatographia* 55(1-2): 5-12.

Lindström B, Ericsson O, Alvan G, Rombo L, Ekman L, Rais M, Sjöqvist F. 1985. Determination of chloroquine and its desethyl metabolite in whole blood: an application for samples collected in capillary tubes and dried on filter paper. *Ther Drug Monit* 7(2): 207-210.

Suppliers

5 Prime → 3 Prime
(see **Eppendorf – 5 Prime**)

ABgene
Web: www.abgene.com

Amersham Biosciences
(also referred to as **Amersham**,
Pharmacia, and **Pharmacia Biotech** in this
manual)
Web: www.amershambiosciences.com

Applied Biosystems
Web: www.appliedbiosystems.com

ATCC
Web: www.atcc.org

Axis-Shield PoC AS
Web: www.axis-shield-poc.com

Bachem
Web: www.bachem.com

Baxter Healthcare
(referred to as **Baxter/Fenwal** in this
manual)
Web: www.baxter.com

BD Biosciences
Web: www.bdbiosciences.com

BD Immunocytometry Systems
Web: www.bdbiosciences.com

BD Labware
Web: www.bdbiosciences.com

BD Vacutainer Systems
Web: www.bd.com/vacutainer

BDH Laboratory Supplies
Web: www.bdh.com

Beckman Coulter
(referred to as **Beckman** in this manual)
Web: www.beckmancoulter.com

Becton Dickinson
Web: www.bdbiosciences.com

Billups-Rothenberg
Web: www.brincubator.com

Bio-Rad Laboratories
Web: www.bio-rad.com

BOC Specialty Gases
Web: www.boc.com

Brinkmann Instruments
Web: www.brinkmann.com

Calbiochem
Web: www.emdbiosciences.com

Cambrex
Web: www.cambrex.com

Clontech
Web: www.bdbiosciences.com/clontech/

Cole-Parmer
Web: www.coleparmer.com

Corning Life Sciences
Web: www.corning.com/lifesciences/

Dako Corp
Web: www.informagen.com

Drummond Scientific
Web: www.drummondsci.com

Eppendorf
Web: www.eppendorf.com

Eppendorf – 5 Prime
(referred to in this manual as **5 Prime → 3 Prime**)
Web: www.5prime.com

Flow Incorporated
Web: www.malariatest.com

Fluka
Web: www.sigma-aldrich.com/Brands/Fluka_Riedel_Home.html

Fresenius France
Web: www.biam2.org

Gentra Systems

Web: www.gentra.com

Gibco

Web: www.invitrogen.com

HCM-Hygenic Corporation

products available from:

Web: www.e-dental.com/buyersguide/

HD supplies

Web: <http://www.tcsbiosciences.co.uk>

ICN Biomedicals

Web: www.icnbiomed.com

Invitrogen

(also referred to as **Gibco** in this manual)

Web: www.invitrogen.com

Iwaki

products available from

Web: www.bibby-sterilin.com

Janssen Pharmaceutica

Web: www.janssenpharmaceutica.be

Joseph Long, Inc.

Phone: 1-973-759-5331

Kahn Paper

Web: www.karnpaper.com

Kontes

Web: www.kimble-kontes.com

Leigh Labs

Web: www.leighlabs.com

Leo Pharma

Web: www.leo-pharma.com

Lillie Glassblower

Phone: +1-404-436-8959

Mabtech

Web: www.mabtech.com

Merck

Web: www.merck.com

Millipore

Web: www.millipore.com

Miltenyi Biotec

Web: www.miltenyibiotec.com

Molecular Dynamics

(see also Amersham Biosciences)

Web: www.mdyn.com

Molecular Probes

Web: www.probes.com

Moravek Biochemicals

Web: www.moravek.com

Nalge Nunc International

(referred to as **Nalgene** and **Nunc** in this manual)

Web: www.nalgenunc.com

Narishige

Web: www.nuhsbaum.com

Nikon

Web: www.nikon-instruments.com

Novakemi

Web: www.novakemi.se

Olympus

Web: <http://microscope.olympus.com>

PerkinElmer

(referred to as **Wallac** in this manual)

Web: las.perkinelmer.com

Pharmacia (see Amersham Biosciences)

Pharmacia Biotech (see Amersham Biosciences)

Promega

Web: www.promega.com

Qiagen

Web: www.qiagen.com

R&D Systems

Web: www.rndsystems.com

Roche

Web: www.roche.com

Roche Applied Science

Web: www.roche-applied-science.com

Savant Speed Vac Systems

Web: www.gmi-inc.com/Products/Savant

Sigma (see Sigma–Aldrich)

Sigma-Aldrich

Web: www.sigmaaldrich.com

Skatron Instruments

Web: www.skatron.com

Sorvall

Web: www.sorvall.com

Sterilin

Web: www.bibby-sterilin.com

Stratagene

Web: www.stratagene.com

Sutter Instrument

Web: www.sutter.com

Syntesome

products available from

Web: www.lectinity.com

Techne

Web: www.techne.com

Terumo

Web: www.terumo.co.jp

Thermo Electron

Web: www.thermo.com

TomTec Cell Harvester

Web: www.tomtec.com

Turner BioSystems

Web: www.turnerbiosystems.com

Vector Laboratories

Web: www.vectorlabs.com

Wallac (see PerkinElmer)

Wardley

Web: www.wardleyfish.com

Waters

Web: www.waters.com

Whatman

Web: www.whatman.com

World Precision Instruments

Web: www.wpiinc.com

ZeptoMetrix

Web: www.zeptometrix.com

Index

- abbreviations, xviii
- acidic phenol, use in RNA extraction, 163-166
- acridine orange, 17
- adhesion medium, formulation, 48
- adhesion of PRBC to
 - immobilized hyaluronic acid, 51-52
 - immobilized receptors, 48-50
- AE, DNA elution buffer, formulation, 224
- agarose-formaldehyde gel
 - formulation, 164
 - use in visualizing RNA, 167-168
- agglutination assay using trophozoite-infected erythrocytes, 61-62
- alanine synchronization of infected erythrocytes, 30
- Albumax complete medium, formulation, 1-2
- alternative CHO and L cell medium,
 - formulation, 37
- alternative MCM, formulation, 2
- amodiaquine, postdose drug level
 - monitoring, 230
- amplification
 - in TRAP assay, 213-214
 - of *msp1*, *msp2*, and *glurp* genes, 216
 - of nucleotide repeat polymorphisms of cytokine genes, 155
 - of phage libraries, 180
- Amplification Refractory Mutation System (ARMS) PCR, 157
- Anopheles* spp., rearing, 118
- Anopheles stephensi*, rearing, 101-102
- antibodies
 - analysis by flow cytometry, 67-68
 - binding and eluting from PRBC surface, 73-74
 - detection on erythrocyte surface, 61-68
 - labelling, 67
 - selection on immobilized antigen or peptide, 139
 - staining of erythrocyte membranes, 135-137
- antigens
 - crude parasite antigen for coating, 142
 - detection by flow cytometry, 65-66
- aphidicolin, arrest of *P. falciparum* trophozoites, 9
- ARMS (Amplification Refractory Mutation System) PCR, 157
- assays
 - binding, 43-44
 - CAT, 198-202
 - cytoadhesion, 37-52
 - exflagellation, 109
 - growth inhibition, 83-86
 - hemozoin formation, 90-92
 - ³H-hypoxanthine incorporation, 87-89
 - in vitro reinvasion, 83-86
 - invasion, 113
 - luciferase, 198-202
 - PfTRAP, 212-214
 - sporozoite invasion, 112-114
 - transmission blocking, 108-111
 - TRAP, 212-214
- AT-rich genomes, PCR, 182
- autoradiography, 128
- binding
 - antibodies on PRBC surface, 73-74
 - assays, 43-44
 - fluorescent receptors, 46-47
 - PRBC to placental tissue, 58-60
- binding medium, formulation, 37, 58
- BL21 competent cells, as transformation host, 196-197
- blocking buffer, formulation, 140
- blood
 - collection, 4, 221, 222
 - cryopreservation, 5
 - establishment of cultures from, 4-7
 - estimation of infection rate, 20
 - sampling, 221-230
 - staining with acridine orange, 17
 - staining with Giemsa, 18-19
 - see also: erythrocytes
- blood feed of mosquitoes
 - equipment, 100-101
 - procedure, 103-104
- blood group A, binding to PRBC, 46-47
- breaking buffer (BrB), formulation, 29
- buffers, formulations
 - blocking buffer, 140
 - breaking buffer (BrB), 29
 - buffer B, 212
 - cell lysis buffer (CLB), 189
 - coating buffer, 135, 137, 138, 140
 - denaturation buffer, 193
 - DNA buffer, 153
 - DNA elution buffer AE, 224
 - electrophoresis buffer, 164, 167

- enzyme substrate buffer, 141
- erythrocyte lysis buffer, 188
- fixation buffer, 77, 81
- fixation storage buffer, 77
- hybridization buffer, 189
- incomplete Cytomix, formulation, 198, 203, 205
- labelling buffer, 123
- loading buffer, 215
- lysing buffer, 153
- lysis buffer, 161
- NETT buffer, 126
- NETT buffer 1, 129
- NETT buffer 2, 129
- neutralization buffer, 193
- PAGE, 159
- phosphate buffer for Giemsa stain, 18
- prehybridization/hybridization buffer, 189
- RNA loading buffer, 164
- running buffer, 131
- sample buffer, 131
- SDS–PAGE sample buffer, 124, 129
- TBE buffer, 189, 215
- TE buffer, 155, 161, 164, 188, 205
- TEN buffer, 199
- TH-buffer, 135
- transfer buffer, 131
- TRAP, 212
- Tris/borate buffer (TBE), 189, 215
- Tris-EDTA buffer (TE), 164
- Tween buffer, 140
- washing buffer (WB), 29, 123
- buffy coat, extraction of DNA, 153-154
- C32-melanoma cells, cytoadhesion, 45
- candle-jar culture method of Trager-Jensen, 1-3
- CAT assay, 198-202
- cDNA
 - insertion, propagation, and amplification in phage, 179-181
 - library construction, 176-177
 - synthesis, 178
- cell lines
 - binding assays, 43-44
 - culturing, 41
 - cytoadhesion, 45
 - freezing, 40
 - thawing, 39
- cell lysis buffer (CLB), formulation, 189
- chloramphenicol acetyl-transferase (CAT) assay, 198-202
- chloroquine
 - as control drug in assays, 87-89
 - postdose drug level monitoring, 230
- CHO cells
 - culture methods, 41
 - cytoadhesion, 45
 - medium formulation, 37
- chondroitin sulfate A as surface receptor, 49
- chromosomes
 - separation and mapping, 188-195
 - staining, 191
- coating buffer, formulation, 135, 137, 138, 140
- complete medium, formulation, 93, 98, 205
- contributors, xi-xvii
- COS cells
 - culture methods, 41
 - medium formulation, 37
- crude parasite antigen, 142, 147
- cryopreservation of
 - blood samples, 5
 - Plasmodium* spp., 12-14
 - Stockholm sorbitol method, 12-13
- cryoprotective solution, 14
- culture medium, formulation, 4
- culture methods for
 - asexual stages of *P. falciparum*, 1-3
 - cell lines, 41
 - erythrocytic stages of *P. chabaudi* in mice, 121
 - erythrocytic stages of *P. vivax*, 10-11
 - gametocytes, 93-99
 - high parasitemia, 8
 - isolates from fresh blood, 5-7
 - transfected parasite lines, 208-210
- cytoadherent parasites, selection with cell lines, 45
- cytoadhesion assays, 37-52
- cytokine-gene polymorphism, detection, 153-158
- cytokines
 - detection, 152
 - mutations in cytokine genes, 157
- Cytomix (incomplete), formulation, 198, 203, 205
- DAB, toxicity warning, 114
- denaturation buffer, formulation, 193
- desalting of synthetic peptides, 148
- DH5 α , as plasmid host, 205
- dissection of blood-fed mosquitoes, 105-106
- DNA
 - buffer, formulation, 153
 - constructs for transfection, 198, 206-207
 - elution buffer AE, formulation, 224
 - extraction from human blood samples in EDTA tubes, 153-154, 227

DNA (*continued*)

- extraction from human blood samples on filter paper, 224-226, 228-229
- extraction from *P. falciparum*, 161-162
- preparation for PFGE, 189-190
- staining, 65
- see also: cDNA; genomic DNA
- drug-induced growth inhibition, assay for, 87-89
- drug-level monitoring, 230
- Durcupan, 79-80
- Dynabeads, enrichment of parasites, 75-76
- EDTA-trypsin solution, formulation, 40-43
- EDTA-tube samples, DNA extraction from, 227
- EDTA tubes, use for blood collection, 222
- electron microscopy
 - embedding of samples, 79
 - fixation protocols, 77-82
- electrophoresis
 - buffer, formulation, 164, 167
 - conditions for PFGE, 190, 194
 - of RNA, 174
- electroporation of circular plasmid DNA into parasites, 207-208
- ELISA
 - antigens for coating, 142
 - equipment, materials, and reagents, 140
 - procedure, 143
- ELISPOT, 150-151
- elongation in TRAP assay, 213
- eluting antibodies on PRBC surface, 73-74
- embedding of samples for electron microscopy, 79-80
- EMEAS (erythrocyte membrane stains by enzyme linked antibodies), 137
- EMIF (erythrocyte membrane immunofluorescence), 135-136
- endothelial cells
 - binding assays, 43-44
 - cytoadhesion, 45
- enrichment of
 - gametocytes with Percoll, 94
 - parasites with Dynabeads, 75-76
 - PRBC with gelatine sedimentation, 23
 - PRBC with Percoll, 25
 - rosetting parasites with Ficoll-Paque, 53
- enzyme substrate buffer, formulation, 141
- enzymes, recovery and purification, 132
- erythrocyte lysis buffer, formulation, 188
- erythrocyte membrane immunofluorescence (EMIF), 135-136
- erythrocyte membrane protein 1, immunoprecipitation, 126-127

erythrocytes (infected)

- adhesion to immobilized hyaluronic acid, 51-52
- adhesion to immobilized receptors, 48-50
- alanine synchronization, 30
- antibodies, detection of, 61-68
- binding and eluting of antibodies, 73-74
- binding fluorescent receptors, 46-47
- binding to placental tissue, 58-60
- enrichment by gelatine sedimentation, 23
- enrichment with Percoll, 25
- estimation of infection with *P. falciparum*, 20
- estimation of infection with *P. vivax*, 10
- fixation for immuno-electron microscopy, 81-82
- growth inhibition assay, 83-84
- immunoglobulin-binding, 69-76
- in vitro reinvasion assay, 83-84
- isolation from placental tissue, 56-57
- membrane immunofluorescence, 135-136
- membrane staining, 137
- metabolic labelling, 128
- micro-agglutination by sera, 63-64
- placenta
 - binding erythrocytes to, 58-60
 - isolating erythrocytes from, 56-57
- purification and synchronization of stages, 23-33
- ³⁵S-methionine-labelling, 128
- separation with Percoll/sorbitol gradient, 26
- serum micro-agglutination, 63-64
- serum protein-binding, 69-76
- sorbitol synchronization, 24
- surface iodination, 123
- see also: blood
- Escherichia coli*
 - BL21 competent cells, as plasmid host, 196-197
 - DH5 α , as plasmid host, 205
 - NM522, as phage host, 179
 - XL10-Gold, as plasmid host, 205
- ethidium bromide, toxicity warning, 10, 46, 67, 71, 135, 155, 157, 167, 173, 188, 193, 215
- exflagellation assay, 109
- exflagellation-inducing medium, formulation, 93
- extraction of
 - blood from filter-paper samples, 223
 - DNA from human blood samples, 153-154
 - surface-radiolabelled proteins, 124

- Ficoll-Paque enrichment of rosetting parasites, 53
- field blood sampling, 221-230
- filter paper, blood collection on, 221
- filter-paper samples
 - DNA extraction from, 224-226, 228-229
 - serum extraction from, 223
- finger prick blood sampling, 221-222
- fixation buffers and other solutions, formulations, 77, 81
- fixation protocols using
 - Durcupan, 79-80
 - formaldehyde, 42
 - glutaraldehyde, 136
 - paraformaldehyde, 77-78, 186
 - paraformaldehyde and glutaraldehyde, 81-82
- flow cytometry
 - analysis of plasma antibodies, 67-68
 - analysis of PRBC, 66
 - detection of surface antigens, 65-66
 - growth inhibition assay, 85-86
 - in vitro reinvasion assay, 85-86
- fluorescence rate, calculation, 47, 71
- fluorescent in situ hybridization for *P. falciparum*, 185-187
- fluorescent receptors, binding, 46-47
- formaldehyde-fixation of melanoma cells, 42
- free parasites, recovery, 28
- freezing-medium formulations
 - cell lines, 40
 - Glycerolyte Method, 14
 - Stockholm sorbitol method, 12
- freezing methods
 - asexual *Plasmodium* spp., 12-13
 - cell lines, 40
 - patient isolates, 14
 - PRBC, 117
 - Stockholm sorbitol method, 12
- freezing solution, formulation, 4
- gametocyte culture, 93-99
- gametogenesis, 94
- gas mixture for gametocyte cultivation, 98
- gelatine sedimentation, enrichment of knob-infected erythrocytes, 23
- genomic DNA, extraction from *P. falciparum*, 161-162
- genotyping *P. falciparum* by PCR, 215-219
- giant proteins, resolution, 131
- Giemsa stain of blood films, 18-19
- glurp* (glutamate rich protein), 215-219
- glutaraldehyde-fixation of monolayers, 136
- glycerolyte, 14
- glycine-HCl elution solution, formulation, 73
- growth inhibition assay, 83-86
- growth inhibition by drugs, 87-89
- guanidine thiocyanate
 - toxicity warning, 173
 - use in RNA extraction, 163-166
 - use in simple Northern, 173-175
- ³H-hypoxanthine incorporation assay, 87-89
- haem incorporation in hemozoin, 91
- haptoglobin phenotyping using PAGE, 159-160
- hemozoin formation assay, 90-92
- heparin
 - binding to PRBC, 46-47
 - use in disrupting rosettes, 69-70
- hepatoma cells (HepG2-A16), use in sporozoite invasion assay, 112-113
- Hoechst 33 342 staining, 65
- human
 - DNA from peripheral blood or buffy coat, 153-154
 - peripheral blood mononuclear cells, preparation, 145-146
- HUVEC cells
 - culture methods, 41
 - cytoadhesion, 45
 - medium formulation, 37
 - thawing, 39
- hyaluronic acid, adhesion of PRBC, 51-52
- hybridization buffer, formulation, 189
- hybridization solution (HS), formulation, 185
- ¹²⁵I-labelled PfEMP1, 126-126
- IFN- γ production, 150-151
- immuno-electron microscopy, 81-82
- immunofluorescence of
 - infected erythrocyte surface, 71-72, 135-136
 - intracellular structures, 138
- immunoglobulin-binding to infected erythrocytes, 69-76
- immunoprecipitation of
 - erythrocyte membrane protein 1, 126-127
 - parasite extracts, 129-130
- in situ hybridization, 185-186
- in vitro reinvasion assay, 83-86
- incomplete Cytomix, formulation, 198, 203, 205
- incomplete RPMI medium, formulation, 93, 185
- infection of
 - monkeys, 115-117
 - mosquitoes, 118-120
- infectivity studies, 96-99
- interleukin ELISPOT, 150-151

- invasion assay, 113
- iodination of erythrocyte surfaces, 123
- isoelectric focusing of proteins, 133-134
- L-cells
 - culture methods, 41
 - medium formulation, 37
- labelling
 - buffer, formulation, 123
 - with ^{125}I , 126-127
 - with ^{36}S , 128
- lactate dehydrogenase assay, 21-22
- lactoperoxidase method of erythrocyte surface iodination, 123
- library construction, 176-177
- loading buffer, formulation, 215
- long-term in vitro cultures of *P. falciparum*, establishment, 4-6
- luciferase assay, 198-202
- lysing buffer, formulation, 153
- lysis buffer, formulation, 161
- M solution, formulation, 185
- MAB 36 for sporozoite detection, 112-113
- magnetic cell sorting (MACS), 31-33
- malaria culture medium (MCM), formulation, 1-2
- media formulations
 - adhesion medium, 48
 - Albumax complete medium, 1-2
 - alternative MCM, 2
 - binding medium, 37, 58
 - CHO and L cell medium, 37
 - complete medium, 93, 98, 205
 - COS-7 medium, 37
 - culture medium, 4
 - exflagellation-inducing medium, 93
 - freezing medium
 - for cell lines, 40
 - for glycerolyte method, 14
 - for Stockholm sorbitol method, 12
 - HUVEC medium, 37
 - incomplete RPMI, 93, 185
 - L-cell medium, 37
 - malaria culture medium (MCM), 1-2
 - RPMI medium, 38
 - RPMI, incomplete, 185
 - RPMI 1640-HEPES medium, 69
 - TCM medium, 83
 - tissue culture medium, 145
 - washing medium, 4
 - YT/amp medium, 196
- mefloquine, postdose drug level monitoring, 230
- melanoma cells
 - binding assays, 43-44
 - culturing, 41
 - cytoadhesion, 45
 - formaldehyde fixation, 42
 - thawing, 39
- membrane feeding, 109-110, 119
- merozoite surface protein, *msp1* and *msp2*, 215-219
- metabolic labelling of PRBC, 128
- methanol extraction of DNA from filter-paper samples, 228-229
- microagglutination of infected erythrocytes, 63-64
- micromanipulation cloning of *P. falciparum*, 34-36
- monitoring of postdose drug levels, 230
- monkeys
 - infection of mosquitoes, 118-120
 - infection with *Plasmodium* spp., 115-117
- mosquito infectivity studies, 96-99
- mosquito pint, preparation, 109
- mosquitoes
 - blood feed of, 103-104
 - dissection of, 105-106
 - infection of, 118-120
 - rearing, 101-102, 118
- mouse, host for *P. chabaudi* infection, 121-122
- mRNA
 - extraction from pooled total RNA, 176
 - extraction of *var*(DBL-1), 183-184
- msp1* and *msp2* (merozoite surface protein), 215-219
- NaCl, use in thawing frozen parasites, 15
- NETT buffer, formulation, 126
- NETT buffer 1, formulation, 129
- NETT buffer 2, formulation, 129
- neutralization buffer, 193
- neutralization solution, formulation, 73
- nitro blue tetrazolium solution, formulation, 21
- NM522, as phage host, 179
- non-detergent sulphobetaines, use in protein purification, 132
- Northern blotting, 173-175
- nucleotide polymorphisms of cytokine genes, amplification of, 155-156
- oligonucleotides
 - sequences of *msp1*, *msp2*, and *glurp* genes, 218-219
 - use in TRAP assay, 212-214
- oocyst production, 100-107
- PAGE (polyacrylamide gel electrophoresis), 159-160

- paraformaldehyde
 - as fixative, 77-78, 81-82, 186
 - toxicity warning, 77, 81
- parasite immunofluorescence (PARIF), 138
- parasitemia
 - culture methods for high parasitemia, 8
 - determination of
 - DNA content, 85-86
 - light microscopy and reticulocyte ocular, 20
 - UV-microscopy, 84
 - monitoring in monkeys, 117
 - volume of medium needed, 8
- parasites
 - chromosomes, separation and mapping, 188-195
 - culture for cDNA library construction, 176-177
 - enrichment using
 - Ficoll-Isopaque, 53
 - Dynabeads, 75-76
 - separation from red blood cells
 - free parasites, 28
 - semi-permeable parasites, 29
 - transfected lines, culture, 208-210
- PARIF, 138
- PBMC, preparation, 143
- PBS stock solutions, formulation, 140, 198
- PCR
 - amplification of nucleotide polymorphisms of cytokine genes, 155-156
 - Amplification Refractory Mutation System (ARMS) PCR, 157
 - genotyping *P. falciparum*, 215-219
 - master mix, formulation, 157, 183
 - of AT-rich genomes, 182
 - see also: RT-PCR
- PECAM-1/CD31, binding to PRBC, 46-47
- peptide/BSA, as coating antigen, 142
- peptides, desalting, 148
- Percoll
 - 60% solution, formulation, 25, 141
 - enrichment of gametocytes, 94
 - enrichment of late-stage infected erythrocytes, 25
 - preparation of parasite antigens, 147
- Percoll/sorbitol gradient, separation of *P. falciparum* mature stages, 26
- peripheral blood, extraction of DNA, 153-154
- peripheral blood mononuclear cells (PBMC), preparation, 143
- PFGE (pulsed-field gel electrophoresis), 188-195
- PfTRAP (telomere repeat amplification in *P. falciparum*), 212-214
- phage, use for cDNA synthesis, 178-181
- Pharmacia kits for cDNA synthesis, 178-181
- phenazine ethosulphate solution, formulation, 21
- phosphate buffer for Giemsa stain, formulation, 18
- placental malaria
 - binding of *P. falciparum*, 58-60
 - isolation of *P. falciparum*, 56-57
- plasma antibodies, analysis by flow cytometry, 67-68
- Plasmodium chabaudi*, infection in mice, 121-122
- Plasmodium coatneyi*, freezing methods, patient isolates, 14
- Plasmodium cynomolgi*, freezing methods, patient isolates, 14
- Plasmodium falciparum*
 - alanine synchronization, 30
 - antigens, measurement of antibodies to, 61-62
 - arrest with aphidicolin, 9
 - cDNA library construction, 176-177
 - chromosomes, separation and mapping, 188-195
 - clones, expansion, 35-36
 - culture methods for
 - blood isolates, 4-7
 - erythrocytic asexual stages, 1-11
 - established cultures, 205-206
 - gametocytes, 93-99
 - high parasitemia cultures, 8
 - DNA extraction, 161
 - enzymes, recovery, 132
 - estimation of infection rate, 20
 - expansion of clones, 35-36
 - fluorescent in situ hybridization, 185-187
 - freezing methods, 14
 - gametocyte culture, 93-99
 - genotyping by PCR, 215-219
 - giant proteins, resolution of, 131
 - hemozoin formation assay, 90-92
 - infection rate estimation, 20
 - long-term in vitro cultures, 4-6
 - micromanipulation cloning, 34-36
 - native proteins, recovery, 132
 - oocyst production, 100-107
 - RNA extraction, 163-172
 - separation of stages with Percoll/sorbitol gradient, 26
 - sorbitol synchronization, 24

Plasmodium falciparum (continued)

- sporozoite production, 100-107
- synchronization, 24, 30
- telomere repeat amplification, 212-214
- thawing methods, 16
- transfection within erythrocytes, 198-204
- transfection of asexual stage, 205-211
- var*- gene domains, expression in bacteria, 196-197
- var*(DBL-1) mRNA, RT-PCR, 183-184
- Plasmodium falciparum*-infected RBC
 - adhesion to
 - immobilized hyaluronic acid, 51-52
 - immobilized receptors, 48-50
 - binding and eluting surface antibodies, 73-74
 - binding of fluorescent receptors, 46-47
 - binding to placental tissue, 58-60
 - counting bound PRBC, 59
 - counting rosettes, 54
 - freezing PRBC, 117
 - membrane immunofluorescence, 135-136
 - metabolic labelling, 128
 - micro-agglutination by sera, 63-64
 - placenta, isolation from, 56-57
 - serum proteins, detection of, 71-72
 - surface, studies of, 123-127
 - surface antigens, detection of, 65-66
- Plasmodium fragile*, freezing methods, patient isolates, 14
- Plasmodium knowlesi*, freezing methods, patient isolates, 14
- Plasmodium* spp.
 - freezing methods, 12-13
 - infection of monkeys with, 115-117
 - thawing methods, 12-13
- Plasmodium vivax*
 - culture methods for erythrocytic asexual stages, 10-11
 - freezing methods, patient isolates, 14
- polyacrylamide-agarose composite gels, 131
- polyacrylamide gel electrophoresis (PAGE), 159-160
- polymerase chain reaction (PCR), see: PCR
- polymorphisms, cytokine-gene, 153-158
- pooter, description, 100
- postdose drug level monitoring, 230
- potassium ferricyanide, toxicity warning, 91
- prehybridization/hybridization buffer, formulation, 189
- prehybridization/hybridization fluid, formulation, 165
- primer mix, formulation, 157
- probe removal solution, formulation, 165

- proguanil, postdose drug level monitoring, 230
- propidium iodide (PI) staining, 85-86
- protein A–Sepharose, capacity, 127
- protein purification, 132-133
- pulsed-field gel electrophoresis (PFGE), 188-195
- purification of
 - erythrocytic stages, 23-33
 - gametocytes, 94
 - proteins, 132-133
- pyridine, toxicity warning, 91
- pyridine-hemochrome method for measuring haem incorporation in hemozoin, 91
- pyrimethamine, use in transfection of *P. falciparum*, 199-200
- receptors, adhesion of PRBC, 48-50
- red blood cells, see: erythrocytes
- reinvasion assay, 83-86
- resolution of giant proteins, 131
- restriction enzyme digestion of chromosomes, 191-192
- reverse transcriptase PCR (RT-PCR), see: RT-PCR
- reversion of rosettes, 54-55
- RNA
 - electrophoresis, 174
 - extraction from *P. falciparum*, 163-172
 - extraction of mRNA from pooled total RNA, 176
 - measurement, 167-168
 - staining, 65
 - visualization, 167-168
- RNA loading buffer, formulation, 164
- rosettes
 - reformation after stripping, 69-70
 - reversion, 54-55
- rosetting assays, 37-52
- rosetting parasites, enrichment using Ficoll–Isopaque, 53
- Rotofor cells, use in protein purification, 133-134
- RPMI, formulation, 38
- RPMI (incomplete), formulation, 185
- RPMI 1640–HEPES medium, formulation, 69
- RT-PCR
 - detection of cytokines, 152
 - detection of *var*(DBL-1) mRNA, 183-184
 - use in micromanipulation cloning, 35
 - see also: PCR
- running buffer, formulation, 131
- ³⁵S-methionine-labelling of *P. falciparum* ring stages, 128

- safety precautions for working with
 - pathogens, 97-98, 113
- salivary glands, dissection, 106
- sample buffer, formulation, 131
- saturated 2-butanol, 189
- SDS-PAGE sample buffer, formulation, 124, 129
- semi-intact cells, extraction, 29
- separation of stages with Percoll/sorbitol gradients, 26
- serum extraction from filter-paper samples, 223
- serum micro-agglutination of infected erythrocytes, 63
- serum protein-binding to infected erythrocytes, 69-76
- short-term cultivation of *P. falciparum*, 7
- sodium acetate solution, formulation, 163
- sodium chloride, use in thawing frozen parasites, 15
- sodium citrate, use in disrupting heparin-resistant rosettes, 69
- solubilization/extraction of surface-radiolabelled proteins, 124-125
- Solution D for RNA extraction, formulation, 163
- sorbitol method of thawing cultures, 16
- sorbitol/Percoll gradient, separation of *P. falciparum* mature stages, 26
- sorbitol synchronization of infected erythrocytes, 24
- Southern hybridization of chromosomes, 191, 194
- sporozoite
 - invasion assay, 112-114
 - production, 100-107
 - visualization, 114
- SSC solution, formulation, 164, 174, 188
- staining
 - chromosomes, 191
 - DNA, 65
 - erythrocyte membranes, 136, 137
 - parasite cultures, 17-22, 99
 - RNA, 65
 - surface immunofluorescence, 65, 136
- stains
 - acridine orange, 17
 - Giemsa, 18-19
 - Hoechst 33 342, 65
 - propidium iodide, 85-86
 - thiazole orange, 65
- stimulation index, 149
- Stockholm sorbitol method, 12-13
- Stratagene
 - Gigapack packaging extracts, 179
 - kits, 176
- sulfadoxine, postdose monitoring, 230
- suppliers, 231-233
- surface antigens
 - detection by flow cytometry, 65-68
 - solubilization/extraction, 124-125
- surface immunofluorescence
 - antibodies for, 74
 - staining, 65, 136
- surface iodination of erythrocytes, 123
- synchronization of *P. falciparum* using
 - alanine, 30
 - sorbitol, 24, 90
- synthetic peptides, desalting, 148
- T-cell proliferation, 149
- TBA (transmission blocking assay), 108-111
- TBE buffer, formulation, 173, 189, 215
- TCM, formulation, 83
- TE buffer, formulation, 155, 161, 164, 188, 205
- telomere repeat amplification in *P. falciparum* (PfTRAP), 212-214
- TEN, formulation, 199
- TH, formulation, 2, 141
- TH-buffer, formulation, 135
- thawing methods
 - for asexual *Plasmodium* spp., 12-13
 - for cell lines, 39
 - for gametocytes, 98
 - for glycerolyte-frozen parasites, 15
 - sorbitol method, 16
 - Stockholm sorbitol method, 12-14
- thawing solution, formulation, 4
- thiazole orange (TO) staining for flow cytometry, 65
- tissue culture medium, formulation, 145
- tissue fixation for transmission electron microscopy, 77-82
- TNT solution, formulation, 185
- toxicity warnings
 - DAB, 114
 - Durcupan, 79
 - ethidium bromide, 10, 46, 67, 71, 135, 155, 157, 167, 173, 188, 193, 215
 - guanidine thiocyanate, 173
 - paraformaldehyde, 77
 - potassium ferricyanide, 91
 - pyridine, 91
- Trager-Jensen candle-jar method, 1-3
- transfected parasites, culture, 208-210
- transfection of *P. falciparum*, 198-211

- transfer buffer, formulation, 131
- transmission blocking assay (TBA), 108-111
- transmission electron microscopy, fixation protocols, 77-82
- TRAP (telomere repeat amplification) assay, 213-214
 - buffer, formulation, 212
- Tris/borate buffer (TBE), formulation, 189, 215
- Tris-buffered Hank's solution, formulation, 2
- Tris-EDTA buffer (TE), formulation, 164
- trophozoites
 - agglutination assay, 61-62
 - arresting growth with aphidicolin, 9
 - selection by magnetic cell sorting, 31-33
 - synchronization with alanine, 30
- TSE, formulation, 193
- Turk, formulation, 145
- Tween buffer, formulation, 140
- Tween wash, formulation, 141
- UV-microscopy for analysis of percent parasitemia, 84
- var*- gene domains, expression in bacteria, 196-197
- var*(DBL-1) mRNA, RT-PCR, 183-184
- washing buffer (WB), formulation, 29, 123
- washing medium, formulation, 4
- Western blotting, antibodies for, 74
- XL10-Gold, as plasmid host, 205
- YT/amp medium, formulation, 196